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Functional Analysis of MicroRNA-10b in Breast Carcinoma: A Dissertation

Charlotte M. Harwood Moriarty
University of Massachusetts Medical School

FUNCTIONAL ANALYSIS OF MICRORNA-10b IN BREAST CARCINOMA

A Dissertation Presented

By

Charlotte M. Harwood Moriarty

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The signatures of the Dissertation Defense Committee signifies
Completion and approval as to style and content of the Dissertation

Arthur M. Mercurio, Thesis Advisor

Victor Ambros, Member of Committee

Roger Davis, Member of Committee

Steen Hansen, Member of Committee

Leslie Shaw, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Sharon Cantor, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

MD/PhD

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List of ABBREVIATIONS

Cdc42	Cell division cycle 42
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
FLT-1	fms-related tyrosine kinase 1 (aka VEGFR-1)
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GDI	Guanine nucleotide dissociation inhibitor
GTPase	Guanine trisphosphatase
KDR	Kinase insert domain receptor (aka VEGFR-2)
LPA	Lysophosphatidic acid
miRNA	MicroRNA
NRP1	Neuropilin-1
NRP2	Neuropilin-2
ORF	Open reading frame
PI3K	Phosphatidyl-inositol 3-kinase
PlGF	Placental growth factor
Rac	Ras-related C3 botulinum toxin substrate 1
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Small interfering RNA
SFM	serum-free medium
TIAM1	T-lymphoma invasion and metastasis 1
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

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FUNCTIONAL ANALYSIS OF MICRORNA-10b IN BREAST CARCINOMA

ABSTRACT

MicroRNAs (miRNAs) represent a class of small noncoding RNAs that regulate gene expression. Recent studies have shown that miRNAs are mis-expressed in various human cancers and that some miRNAs have the potential to act as tumor suppressors or oncogenes. MiR-10b is one miRNA that has been shown to be deregulated in breast cancer. However, current findings regarding miR-10b's role in breast cancer are controversial. MiR-10b was originally reported to be downregulated in breast cancer compared to normal breast tissue. Subsequently, miR-10b was argued to be upregulated in metastatic breast cancer cell lines, acting as a potent pro-metastatic agent via regulation of HOXD10. This report was soon challenged by another group who reported that miR-10b expression in a large patient cohort correlated inversely and significantly with tumor size, grade, and vascular invasion, but did not correlate with development of distant metastases or survival. These latter data suggest that miR-10b may impede specific functions associated with breast cancer progression.

In this thesis, I present my analysis of miR-10b function in breast carcinoma cells, which revealed that it suppresses their migration and invasion. To define a mechanism that accounts for this suppressive function, I identified T-

lymphoma invasion and metastasis 1 (TIAM1), a guanine nucleotide exchange factor for Rac1, as a miR-10b target and demonstrated that miR-10b inhibits TIAM1-dependent Rac1 activation, migration, and invasion. In addition, I identified the VEGF receptor fms-related tyrosine kinase 1 (FLT-1) as a second target of miR-10b and discovered a novel function for FLT-1 in promoting breast carcinoma cell migration and invasion. My results show, for the first time, that Rac activation can be regulated by a specific miRNA and provide a novel mechanism for the regulation of TIAM1 and FLT-1 in breast cancer. These data support the conclusion from clinical data that miR-10b expression correlates inversely with breast cancer progression, and suggest that miR-10b functions to impede breast carcinoma progression by regulating key target genes involved in cell motility.

CHAPTER I

INTRODUCTION

Cell motility is essential to many normal cell processes, including embryonic morphogenesis, wound healing, and immune-cell trafficking (Friedl and Brocker, 2000). The molecular mechanisms of cell migration involve continuous cycling of a series of interdependent processes, beginning with abrogation of cell-cell contacts and protrusion of the leading edge, followed by cell-matrix interaction and formation of focal contacts, the recruitment of surface proteases to extracellular matrix (ECM) contacts and localized proteolysis of the ECM, cell contraction by actomyosin, and finally detachment of the trailing edge (Friedl and Wolf, 2003; Lauffenburger and Horwitz, 1996). These processes are regulated by cell-cell matrix adhesion molecules such as integrins, matrix-degrading enzymes, cell-cell adhesion molecules, and cell-cell communication. In addition, factors within the microenvironment, including chemokines and growth factors such as epidermal growth factor (EGF) and lysophosphatidic acid (LPA), can propel, direct, and regulate cell motility. These motility-inducing factors promote cell migration by promigratory signal transduction via phosphatidyl-inositol 3-kinase (PI3K) and Rho GTPases Rac and Rho (Friedl and Wolf, 2003).

Tumor cells are believed to use similar, if not identical, mechanisms of migration to those that occur in normal cells (Friedl and Brocker, 2000). The

aberrant activation of these motility pathways – a dominance of promigratory events in the absence of counteracting stop signals (Alper et al., 2001; Giannelli et al., 1997) – is an essential feature of the metastatic process (Gupta and Massague, 2006).

The processes involved in carcinoma progression include tumor cell migration and invasion of surrounding tissues, and a “metastatic cascade,” in which a carcinoma cell acquires the ability to escape from the tumor mass, penetrate tumor blood vessels or local lymphatics, disseminate through the vasculature, extravasate from the vessels, colonize distant organs, and expand to form metastases (Liotta et al., 1991). The ability of carcinoma cells to invade surrounding tissues requires altered cell adhesion, loss of cell polarization, cytoskeletal rearrangement, and basement membrane remodeling (Sahai and Marshall, 2002). In preventing metastatic disease, the processes of local invasion and metastatic spread are the most clinically relevant, yet they remain the most poorly understood at the molecular level. Thus, identifying the mechanisms by which tumor cells acquire such invasive and metastatic potential is critical for developing strategies to impair cancer progression in a clinical setting (Chambers et al., 2002).

Proposed mechanisms of metastatic spread include the epithelial-to-mesenchymal transition (EMT), a phenotype switch in which tumors cells with a highly differentiated epithelial morphology assume a migratory and invasive phenotype, and collective migration, in which a group of tumor cells metastasize

together (Christiansen and Rajasekaran, 2006). The EMT involves changes in gene expression, distribution, and function of growth factors, transcription factors, cell-to-cell adhesion molecules, cell-to-ECM adhesion molecules, cytoskeletal modulators, and extracellular proteases - the same elements commonly associated with cancer progression (Thompson et al., 2005). These critical elements lead to changes in tumor microenvironment, cell adhesion, and the cell signaling pathways that regulate cell motility (Christofori, 2006; Liotta and Kohn, 2001). Significant overlap exists between migration and invasion pathways. An understanding of the ability of a carcinoma cell to acquire the migratory and invasive capabilities that lead to metastatic disease is a key area for identifying mechanisms of cancer progression and preventing metastasis in a clinical setting.

MICRORNAs

MicroRNAs (miRNAs) are a recently discovered class of regulatory RNAs that provide an attractive model for global gene regulation during breast cancer progression (Tavazoie et al., 2008). MiRNAs are small, single stranded, noncoding RNAs that function to regulate protein expression levels from protein coding RNAs (Bartel, 2004). MiRNAs were discovered following the observation that the *lin-4* gene, essential for development of the nematode worm *C. elegans*, does not encode protein. Instead the gene product is a small RNA that acts as a negative regulator of another critical gene, *lin-14*, by binding to the 3’

untranslated region (UTR) of the *lin-14* transcript, and thereby inhibiting its translation (Lee et al., 1993; Wightman et al., 1993).

MiRNAs are transcribed from the genome as primary transcripts that are extensively processed to produce mature, functional miRNAs (Esquela-Kerscher and Slack, 2006) (Figure 1). In humans, miRNA genes are transcribed by RNA polymerase II to produce capped and polyadenylated primary transcripts, termed 'pri-miRNAs' (Lee et al., 2002). These miRNA precursors contain stem-loop structures in which the bases in each molecule pair internally and often imperfectly. The stem-loop structures are necessary for correct processing by the RNAse III endonuclease Drosha and its co-factor DGCR8. Drosha and DGCR8 cleave the loose ends of pri-miRNAs near the base of the stem loop to liberate ~60-nucleotide pre-miRNA stem-loops (Basyuk et al., 2003; Cai et al., 2004; Denli et al., 2004; Gregory et al., 2004; Lee et al., 2003; Lee et al., 2002; Lee et al., 2004; Zeng and Cullen, 2003). Pre-miRNAs are exported from the nucleus by exportin and Ran-GTP, and further processed in the cytoplasm by another RNAse III enzyme, Dicer, to ~22 nt double-stranded siRNA-like imperfect duplexes composed of the mature miRNA and a similar-sized fragment obtained from the opposing arm of the pre-miRNA (Bagga et al., 2005; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). The fragment from the opposing arm, termed the miRNA* strand, is often peeled away and degraded when the miRNA strand of the miRNA:miRNA* duplex is loaded into the RNA-induced silencing complex (RISC) (Filipowicz et al., 2008; Kim and Kim, 2007).

Prevailing models suggest there are two mechanisms by which miRNAs regulate the function of their target mRNAs. The RISC complex can combine with either the 3'UTR or the open reading frame (ORF) of the target mRNA based on the complementarity of the miRNA sequence to the target. Binding to the 3'UTR seems to require only imperfect complementarity, specifically conserved Watson-Crick pairing to the 5' region of the miRNA centered on nucleotides 2-7, the miRNA "seed" (Bartel, 2009). Binding with insufficient complementarity often results in translational repression of the target mRNA. On the other hand, binding within the ORF requires perfect or near-perfect complementarity, resulting in mRNA cleavage and degradation of the mRNA by Argonaute 2 (Ago2), a catalytic endonuclease component of RISC (Meister et al., 2004). Both mechanisms ultimately result in downregulation of protein expression from target genes. Recent findings indicate rare alternative mechanisms also exist, including targeting to the 5'UTR (Orom et al., 2008) and degradation of target mRNAs having only partial complementarity to their regulatory miRNAs. Although several mechanisms of regulation exist, 3'UTR targeting appears to occur most frequently and most effectively, followed by endogenous ORF targeting, and finally, 5'UTR targeting (Bartel, 2009). Human miRNAs are thought to use primarily the first mechanism of regulation by 3'UTR targeting leading to translational repression (Bartel, 2004; Esquela-Kerscher and Slack, 2006).

Each individual miRNA has the potential to regulate multiple mRNAs (John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003;

Lim et al., 2005; Rajewsky and Socci, 2004; Rehmsmeier et al., 2004). Similarly, each mRNA is believed to be targeted by several different miRNAs (Pillai, 2005). As a result, miRNAs provide an attractive model for global regulation of gene expression. In fact, these regulatory molecules have been implicated in an increasing number of biological processes, including the development and progression of cancer (Calin et al., 2002; Cimmino et al., 2005; Johnson et al., 2005; Michael et al., 2003; Takamizawa et al., 2004). Recent studies have shown that certain miRNAs are deregulated in cancer (Calin and Croce, 2006; Calin et al., 2005; Iorio et al., 2005; Lu et al., 2005), and that they can influence key cell biological processes that affect tumor progression, including migration, invasion, epithelial to mesenchymal transition (Burk et al., 2008; Korpal et al., 2008) and metastasis (Huang et al., 2008; Tavazoie et al., 2008; Zhu et al., 2008). MiR-10b is one such miRNA.

MiR-10b

MiRNA microarray profiling of 76 primary breast tumors and 10 normal breast tissue samples revealed that miR-10b was significantly downregulated in breast cancer, and identified miR-10b as one of 15 miRNAs in a signature that was able to correctly predict the nature of a breast tissue sample (i.e. normal vs. tumor) (Iorio et al., 2005). These results suggested that miR-10b may have a role in breast cancer suppression. However, another group suggested the opposite conclusion two years later, proclaiming miR-10b to be a pro-metastatic miRNA (Ma et al., 2007). In human breast cell lines and mouse xenograft models of

breast cancer, miR-10b was found to be highly expressed only in metastatic cells and to promote cancer cell invasion and metastasis *in vivo* (Ma et al., 2007). Furthermore, it was reported that the metastasis-promoting transcription factor Twist (Yang et al., 2004) binds to the putative miR-10b promoter, thereby enhancing transcription of the *mir-10b* gene. In turn, miR-10b was reported to repress HoxD10 directly by binding to its 3'UTR and inhibiting translation. RhoC, an important mediator of cell migration and metastasis (Bellovin et al., 2006; Clark et al., 2000; Hakem et al., 2005; Kleer et al., 2006; Kondo et al., 2004) that is known to be repressed by HoxD10 (Myers et al., 2002), was reported to be elevated in miR-10b-expressing cells, providing a mechanism for increased cell migration and invasion. Finally, miR-10b expression in a small group of primary breast tumors was reported to correlate significantly with clinical progression (Ma et al., 2007).

Interestingly, the above findings on miR-10b and metastasis were challenged soon after when a third group found no significant association between miR-10b expression and metastasis or prognosis by real-time PCR (Gee et al., 2008). Using fresh frozen samples and long term follow-up data from 219 patients with early breast cancer, they reported lower expression levels of miR-10b in patients without metastases than in normal breast tissue; supporting the original finding that miR-10b is downregulated in breast cancer. In direct contradiction to the Ma et al. study (2007), miR-10b expression was reported to correlate inversely with tumor size and grade. Furthermore, there was no

significant correlation between miR-10b and metastasis outcome (Gee et al., 2008). Collectively, these studies suggest that much work needs to be done before drawing conclusions about the function of miR-10b in breast cancer.

While miRNAs such as miR-10b have the capacity to regulate a large number of genes simultaneously, disrupting the miRNA regulation of a single target can have significant phenotypic effects (Bartel, 2007). Among the predicted targets of miR-10b in TargetScan, a computational algorithm that predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer 3'UTR sites that match the seed region of each miRNA (Lewis et al., 2005), two genes - TIAM1 and FLT-1 - have been previously implicated in breast cancer progression and metastasis. It is possible that these genes may promote breast carcinoma progression as part of a miR-10b-regulated pathway.

TIAM1

The guanosine triphosphatases (GTPases) Rho, Rac (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (cell division cycle 42) are members of the Rho family, a subset of the Ras superfamily (Rossman et al., 2005; Schmitz et al., 2000).These proteins function as molecular switches and play a role in many cellular processes, including regulation of the actin cytoskeleton, cell cycle progression, gene transcription, and cellular adhesion (Ridley, 2001). In addition, mutations and aberrant regulation of these proteins have been implicated in tumorigenesis (Advani and Pendergast, 2002; Engers et al., 2000; Kourlas et al.,

2000; Malliri et al., 2002; Sahai and Marshall, 2002). Rho family proteins are active only when bound to GTP and although all possess weak intrinsic GTPase activity, their activity level is tightly controlled by three general classes of proteins: GTPase-activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDIs), and guanine nucleotide exchange factors (GEFs) (Figure 2). GAPs function to enhance the ability of the GTPase to hydrolyze GTP to GDP, thereby controlling the rate of movement from the active conformation to the inactive conformation. GDIs serve as an anchor by forming a large complex with the Rho protein helping to prevent diffusion within the membrane and into the cytosol, allowing for spatial control of Rho activation. Finally, GEFs facilitate the exchange of GDP for GTP, thereby enhancing Rho activation (Ellenbroek and Collard, 2007).

One of the most studied GEFs that regulates Rho family GTPases is T-lymphoma invasion and metastasis gene 1 (TIAM1). TIAM1 was originally identified by proviral tagging and *in vitro* selection for invasiveness from murine lymphoma cells in 1994 (Habets et al., 1994). The human homologue was identified one year later (Habets et al., 1995). TIAM1 is a member of Dbl family of GEFs and acts as an exchange factor for the Rho GTPases Rac1 and Cdc42, and to a lesser extent, RhoA (Michiels et al., 1995). TIAM1 induces extensive cellular ruffling in fibroblasts via Rac1, in a process independent of RhoA (Michiels et al., 1995). In addition, TIAM1 induces invasion of T-lymphoma cells into a fibroblast monolayer, as do constitutively active Rac1 and Cdc42. RhoA

potentiates invasion and is required for an invasive phenotype, but is not sufficient (Stam et al., 1998). Other studies have shown that RhoA can also function to impede cell invasion in invasive breast carcinoma due to a reciprocal relationship between RhoA and Rac1 activation (Simpson et al., 2004). Furthermore, TIAM1 overexpression in neuroblastoma cells promotes cell spreading and adhesion, as well as neurite outgrowth. These events are dependent on Rac1 and the cells fail to respond to processes driven by Rho, such as LPA-induced neurite retraction and cell rounding. Collectively, these findings suggest that TIAM1 plays a role in cytoskeletal rearrangement during cell migration and that Rho may oppose TIAM1-induced activation of Rac1. In fact, TIAM1-induced effects are abrogated by co-expression of constitutively active Rho, suggesting a delicate balance between Rac and Rho signaling (Leeuwen et al., 1997).

Several studies have suggested that TIAM1 induces cellular adhesion, as opposed to cellular migration. For example, TIAM1 is reported to concentrate at adherens junctions in canine kidney epithelial cells. Ectopic expression of TIAM1 or constitutively active Rac1 inhibits scattering of these cells by increasing E-cadherin-mediated cell-cell adhesion and polymerizing actin at cell-cell contacts. In Ras-transformed epithelial cells, expression of TIAM1 reverses the fibroblast-like morphology to an epithelial-like phenotype (Hordijk et al., 1997). Similarly, TIAM1 expression in renal cell carcinoma cell lines correlates inversely with invasive potential, and overexpression of TIAM1 or constitutively active Rac1 in

these cells inhibits cell migration by promoting E-cadherin-mediated adhesion (Engers et al., 2001; Engers et al., 2000). Thus, TIAM1's effects are thought to be cell-type specific, increasing motility in mesenchymal cell types and increasing cellular adhesion in epithelial cells. In addition, TIAM1/Rac-induced cellular response is dependent on the cell substrate (Sander et al., 1998b). For example, on fibronectin and laminin 1, Tiam1/Rac signaling inhibits migration by restoring E-cadherin-mediated cell-cell adhesion. However, on different collagens, formation of E-cadherin adhesions is prevented and expression of Tiam1 promotes cell motility. The status of Rac and Rho activation may also be critical to the effects of TIAM1. As TIAM1 acts as a GEF for both of these GTPases, different levels of activation of Rac and Rho may account for different biologic effects (Leeuwen et al., 1997; Sander et al., 1999).

Recent studies indicate that TIAM1 plays in an important role in the progression of epithelial cancers, especially carcinomas of the colon and breast (Minard et al., 2004; Morikawa et al., 1988a; Morikawa et al., 1988b). Colon cancer cell lines selected for a high migratory phenotype demonstrate increased TIAM1 expression levels and overexpression of TIAM1 in the parental cell line increases cell migration. Furthermore, both populations of cells demonstrate increased metastatic potential in the nude mouse (Minard et al., 2004). Another group reported that colon carcinoma cell lines selected for increased metastatic potential in the nude mouse expressed higher levels of TIAM1 than parental cell lines (Morikawa et al., 1988a; Morikawa et al., 1988b). Collectively, these data

indicate that TIAM1 may play a role in the progression and metastasis of colon cancer.

TIAM1 appears to play a similar role in promoting breast carcinoma progression. In murine breast cancer cells, the cellular adhesion molecule and receptor for hyaluronic acid (HA) binding receptor CD44 binds TIAM1, resulting in an increase in TIAM1-mediated Rac1 activation and cytoskeleton-mediated tumor cell migration (Bourguignon et al., 2000; Underhill et al., 1987). Furthermore, TIAM1 overexpression induces cellular ruffles and loss of intracellular adhesiveness, and increases the migratory and invasive phenotypes of breast cancer cell lines. In human breast carcinomas, a close correlation was observed between increased expression of TIAM1 and the invasiveness of breast tumor cells with the degree of progression of breast tumors (Adam et al., 2001). In support of these findings, TIAM1 expression was reported to correlate with migratory capabilities and metastatic potential in a panel of eleven human breast cancer cell lines (Minard et al., 2004). These results suggest that increased TIAM1 activity or expression may promote breast cancer progression. However, expression of TIAM1 in breast carcinoma metastases remains to be determined.

In breast carcinoma cells, TIAM1 overexpression is likely to constitutively activate the GTPases Rac, Rho, or Cdc42. Activation of these proteins in turn activates downstream signaling pathways capable of promoting cell migration and invasion, critical properties in the development of the metastatic phenotype.

Therefore, future studies elucidating the signaling pathways involving TIAM1 are needed to provide important insights into the progression and metastasis of breast carcinoma.

FLT-1

Another important process in cancer progression and metastasis is angiogenesis, the development of new blood vessels. Blood vessel formation between a tumor and its surrounding tissue is essential for cancer growth, because it provides tumor cells with oxygen and other vital nutrients (Folkman, 1992). Carcinoma cells themselves stimulate angiogenesis via production and secretion of vascular endothelial growth factor (VEGF) (Verheul and Pinedo, 2000). VEGF interacts with vicinal endothelial cell receptors in a paracrine fashion, including FLT-1 (fms-related tyrosine kinase 1, aka VEGFR-1) and KDR (kinase insert domain receptor, aka VEGFR-2), as well as co-receptors neuropilin-1(NRP1) and neuropilin-2 (NRP2), to drive signaling pathways promoting formation of new blood vessels (Shibuya, 2001). VEGF is considered to be an essential factor in cancer progression, because of its positive effects on blood vessel formation (Zachary, 1998). However, recent work has shown that the VEGF receptors FLT-1, NRP1, and NRP2 are also present on the surface of some carcinoma cells, indicating that VEGF may affect tumor cells directly in an autocrine or paracrine manner as well. In fact, VEGF has been demonstrated to function in an autocrine manner in breast carcinoma cells to stimulate signaling

pathways that maintain cell survival (Bachelder et al., 2002). Furthermore, survival of colon carcinoma cells was found to be dependent on a FLT-1-mediated VEGF autocrine signaling pathway (Bates et al., 2003). Such an elaboration of autocrine signaling pathways provides carcinoma cells with a degree of self-sufficiency that increases their probability to progress and possibly become invasive and/or metastatic.

The contribution of VEGF signaling in carcinoma cells to their ability to evade cell death is a significant advance in our understanding of the role of VEGF in cancer biology. Moreover, a novel result that derives from this finding is the involvement of FLT-1 in mediating VEGF survival signaling (Bates et al., 2003). FLT-1 is one of two high affinity receptor tyrosine kinases for VEGF, the second being KDR. VEGF co-receptors NRP1 and NRP2 lack intrinsic signaling properties and are thought to function as co-receptors that enhance VEGF binding to FLT-1 and KDR. FLT-1 demonstrates a higher binding affinity for VEGF in comparison with KDR, but weak tyrosine kinase activity (Waltenberger et al., 1994). FLT-1 was initially believed act as a decoy receptor, leading to negative regulation of VEGF signaling by preventing VEGF binding to KDR. Subsequent studies revealed that FLT-1 plays a critical role in the growth and migration of endothelial cells in both developmental and pathologic angiogenesis (Barleon et al., 1996; Hiratsuka et al., 2001) and that this activity is independent of the tyrosine kinase domain (Hiratsuka et al., 1998). FLT-1 was later identified as a key factor in promoting the migration of monocytes and macrophages,

inflammatory diseases, atherosclerosis, and cancer metastasis (Sawano et al., 2001; Shibuya, 2006). Specifically, FLT-1 expression on bone marrow-derived cells promotes cancer metastases by promoting the infiltration of these cells and inducing matrix metalloproteinase-9 in premetastatic tissues, forming a "premetastatic niche" (Kaplan et al., 2005). Remarkably, inhibition of these FLT-1 positive hematopoietic progenitors using an anti-FLT-1 peptide prevented formation of metastases (Bae et al., 2005).

FLT-1 acts as a receptor for placental growth factor (PlGF) in addition to VEGF. PlGF signals directly through FLT-1 in several cell types, including endothelial cells, macrophages, and tumor cells, thereby acting to promote tumor angiogenesis, tumor growth, and the formation of the premestastatic niche (Fischer et al., 2008). Emerging reports have documented that PlGF might be a prognostic marker for some tumor types, including breast. In fact, PlGF levels in breast cancer are reported to correlate with recurrence, metastasis, and mortality (Parr et al., 2005), and αPlGF therapy has been found to block tumor growth, metastasis, lymphangiogenesis, and angiogenesis (Fischer et al., 2007). Collectively, these findings suggest that FLT-1 signaling represents an important target in the suppression of cancer progression and metastasis.

Recent studies indicate that FLT-1 expression is upregulated in several human carcinoma cell lines, as well as several common tumors, including prostate, breast and colon (Andre et al., 2000; Fan et al., 2005; Jackson et al., 2002; Plate et al., 1994; Price et al., 2001; Speirs and Atkin, 1999). Furthermore,

expression of FLT-1 in various carcinoma cells has been found to correlate with cancer progression, cell survival (Bates et al., 2003), proliferation, migration and invasion (Fan et al., 2005; Lesslie et al., 2006; Wey et al., 2005), and distant metastasis (Hiratsuka et al., 2002). In pancreatic cancer, FLT-1 is reported to mediate EMT via increased activity of the transcription factors Snail, Twist, and Slug (Yang et al., 2006). A recent study in breast carcinoma suggests that FLT-1 enhances cell survival through an internal autocrine signaling pathway (Lee et al., 2007). However, the regulation and function of FLT-1 in breast carcinoma remain largely unknown.

Gene expression profiling of node-negative primary breast tumors revealed that FLT-1 expression is a key component of a ‘poor prognosis gene signature’ that is strongly predictive of a short interval to distant metastases (van 't Veer et al., 2002). This finding is substantiated by the fact that FLT-1 expression, as assessed by immunohistochemistry, correlates with a high risk of metastasis and relapse in both node negative and invasive breast cancer (Ghosh et al., 2008; Meunier-Carpentier et al., 2005; Mylona et al., 2007). Notably, anti-FLT-1 antibody or FLT-1-specific inhibitory peptides suppress tumor growth and metastasis in various models, including breast (Bae et al., 2005; Luttun et al., 2002; Taylor and Goldenberg, 2007; Wu et al., 2006). Collectively, these studies indicate that FLT-1 may promote an aggressive phenotype in human breast cancer, but further studies are necessary to ascertain its function and how it is regulated.

I hypothesize that miR-10b plays a key role in breast cancer progression, in part via the regulation of cell motility through TIAM1 and FLT-1. My thesis work, presented in the following chapters, explores the function of miR-10b in breast cancer, its ability to regulate putative targets TIAM1 and FLT-1 in breast cancer, and the function of FLT-1 in breast cancer.

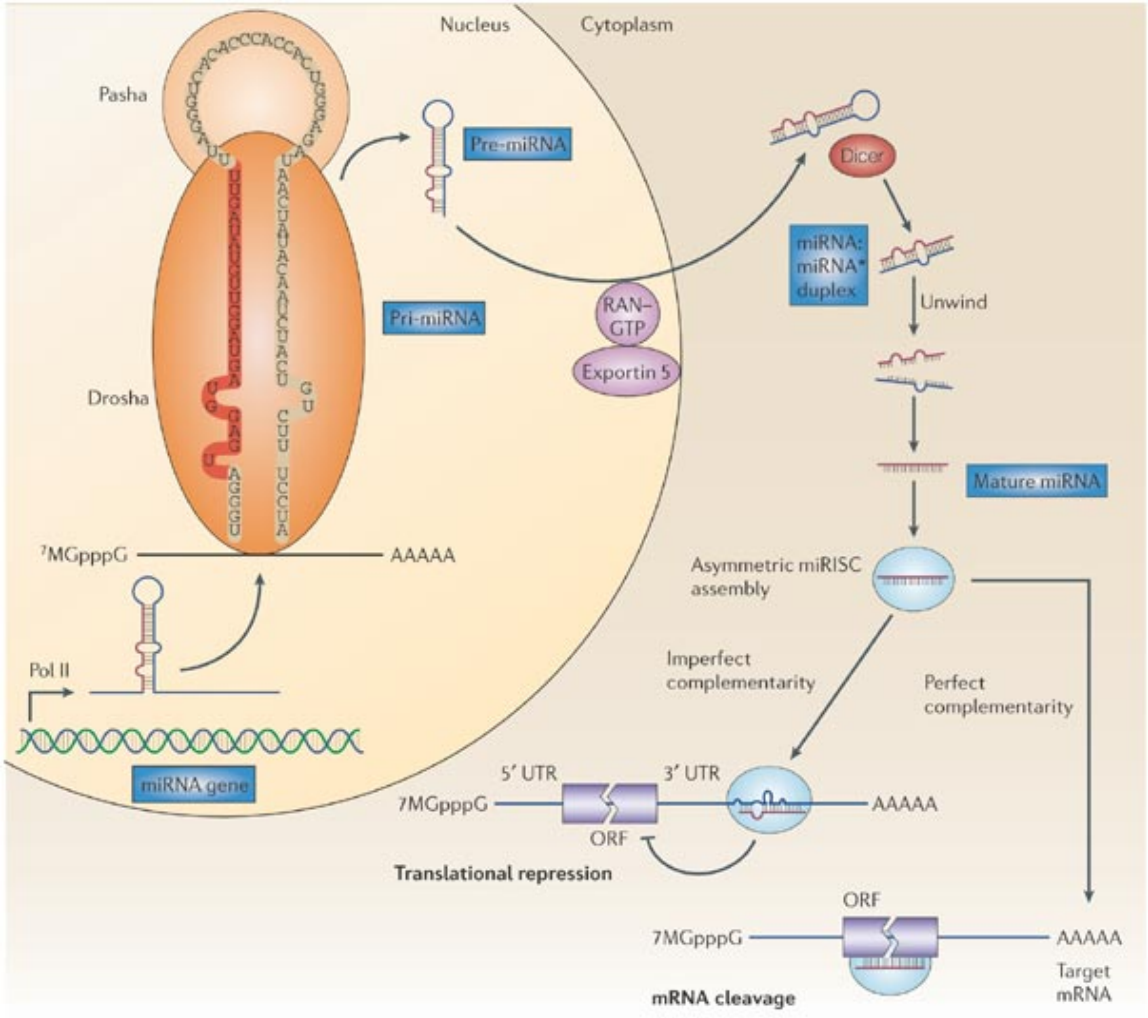

Figure 1. MiRNA biogenesis.

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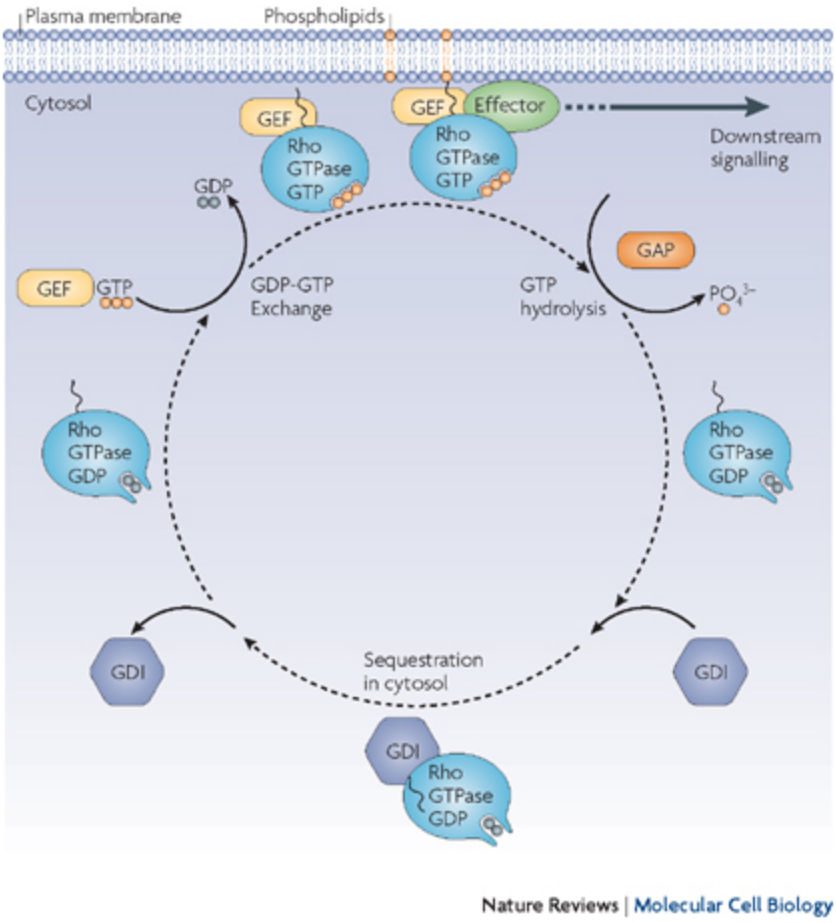

Figure 2. Rho family GTPases and their regulators.

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CHAPTER II

MIR-10B IMPEDES BREAST CARCINOMA CELL MIGRATION and INVASION BY TARGETING TIAM1

Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs that control gene expression by targeting mRNAs and triggering either translational repression or mRNA degradation. Recent evidence has shown that miRNAs are aberrantly expressed in human cancer (Calin and Croce, 2006; Iorio et al., 2005; Lu et al., 2005; Ozen et al., 2008) and that they can affect key cell biological processes that affect tumor progression including migration, invasion, epithelial to mesenchymal transition (Burk et al., 2008; Korpal et al., 2008), and metastasis (Huang et al., 2008; Tavazoie et al., 2008; Zhu et al., 2008). The challenge ahead is to elucidate specific mechanisms by which miRNAs regulate such processes.

MiR-10b is one of 29 miRNAs whose expression has been reported to be significantly deregulated in breast cancer (Iorio et al., 2005). This miRNA achieved prominence because its expression in primary breast tumors was found to correlate with their ability to metastasize, and it was shown to *promote* the migration and invasion of breast carcinoma cells *in vitro* (Ma et al., 2007).

However, this seminal role for miR-10b in breast cancer was challenged recently based on the analysis of miR-10b expression in a large group of patients with early breast cancer (Gee et al., 2008). In this study, miR-10b expression did not correlate with development of distant metastases, recurrence-free survival or distant-relapse-free survival. Instead, miR-10b expression correlated inversely and significantly with tumor size, grade, and vascular invasion. These data infer that miR-10b impedes specific functions associated with breast cancer progression, and they highlight the need for more mechanistic studies.

In this study, I present my analysis of miR-10b function in breast carcinoma cells, which revealed that it *suppresses* their migration and invasion. To define a mechanism that accounts for this suppressive function, I identified TIAM1, a GEF for Rac, as a miR-10b target and demonstrated that miR-10b inhibits TIAM1-dependent Rac activation, migration, and invasion. These data reveal a novel function of miRNAs and they support the conclusion from clinical data that miR-10b expression correlates inversely with breast cancer progression.

Results

To assess the ability of miR-10b to regulate migration and invasion as reported previously, I used two breast carcinoma cell lines (SUM-149PT and SUM-159PT) that are highly motile and invasive, and one (T47D) that is not (Dong et al., 2007; Flanagan et al., 1999; Gordon et al., 2003). Reverse

transcriptase (RT)-PCR analysis revealed that SUM-149PT and SUM-159PT cells lack miR-10b expression but that T47D cells express miR-10b (Fig. 1A). To examine whether exogenous expression of miR-10b influences migration, I used a miR-10b precursor for *de novo* expression in SUM-159PT cells. This precursor is a chemically-modified double-stranded RNA modeled on the sequence of mature miR-10b. For a control, I designed a miR-10b mutant with a single base pair substitution in the seed sequence of the mature strand (Fig. 1B). By introducing mismatch into the critical seed region, binding of the miRNA to its target genes should be reduced or abolished. A non-targeting miRNA was used as an additional negative control. Transient expression of miR-10b in SUM-159PT cells resulted in expression of mature miR-10b, as assessed by RT-PCR (Fig. 1C). The miR-10b mutant, differing from miR-10b by a single base pair, is also detected by the primers used. To examine whether the miR-10b mutant was being expressed at the same level as miR-10b, I used quantitative RealTime PCR to amplify miR-10b and the miR-10b mutant using sequence specific primers and found that the miR-10b mutant was expressed 1.5-fold higher than miR-10b (Fig. 1C), allaying concerns that the miR-10b mutant was expressed at lower levels than miR-10b.

For migration and invasion assays, transwells were coated with 15 µg/ml collagen or 0.5 µg matrigel, respectively. Cells expressing miR-10b, miR-10b mutant, or non-targeting control were then seeded into the upper chamber in serum-free medium and allowed to migrate/invade towards NIH-3T3-conditioned

medium for 4 hours at 37ºC. Expression of miR-10b resulted in a 40% decrease in both migration and invasion as compared to non-targeting control miRNA (Fig. 1D).

To confirm that miR-10b, in contrast to published results, inhibits cell motility and that I was not observing an artifact of the miRNA precursor, I utilized a miR-10b expression vector. This retroviral vector encodes the genomic sequence of the human *miR-10b* gene in the 3' UTR of green fluorescent protein (GFP) and requires that mature miR-10b be generated through endogenous cellular processing. I used this vector to express miR-10b in SUM-159PT cells and confirmed expression of the mature sequence by RT-PCR (Fig. 1E). Ectopic expression of this miR-10b resulted in greater than 50% decrease in both cell migration and invasion in comparison to the empty vector control (Fig. 1E). To further verify this result, miR-10b was expressed in SUM-149PT cells, which resulted in a 40% decrease in cell migration and invasion over a 16 hour period (Fig. 1F). In the previous publication by Ma et al. (2007), the same cell line transduced with the very same expression vector resulted in an *increase* in cell migration and invasion. My culture conditions for the SUM-149PT cells were identical to those published, but I discovered that my migration assays were conducted slightly differently. To account for these differences, I followed the published protocol of using non-coated transwells for migration assays, with 10% serum as the chemoattractant in the lower chamber. In striking contrast to

published results, cells expressing miR-10b exhibited a greater than 3-fold *decrease* in cell migration under these conditions (Fig. 1G).

I next asked whether inhibition of endogenous miR-10b in T47D cells would affect their motility. For this purpose, I designed an antisense oligonucleotide to silence miR-10b. Indeed, expression of antisense miR-10b increased both migration and invasion of T47D cells by approximately 2-fold (Fig. 2).

To understand the mechanisms by which miR-10b represses cell motility, I used computational algorithms to identify miR-10b target genes. The search program TargetScan revealed several predicted targets known to play a role in cell migration and invasion, including T lymphoma invasion and metastasis 1 (TIAM1) and nuclear factor of activated T cells 5 (NFAT5). TIAM1 was of particular interest because its expression correlates with epithelial tumorigenicity, the metastatic potential of human breast cancer cell lines (Minard et al., 2004), and increased breast cancer grade (Adam et al., 2001). The predicted target site for miR-10b is a single 8mer site, comprised of the seed match flanked by both the match at position 8 and the A at position 1 (Lewis et al., 2005). I observed a dramatic reduction in TIAM1 protein levels in both SUM-159PT and SUM-149PT cells expressing miR-10b, as compared to controls (Fig. 3A and 3B). Co-transfection of the miR-10b precursor with miR-10b antisense rescued expression of TIAM1. Conversely, transfection of miR-10b antisense in T47D

cells to silence endogenous miR-10b led to a corresponding increase in TIAM1 protein (Fig. 3C).

To determine whether regulation of TIAM1 expression by miR-10b is direct, I utilized a luciferase reporter gene fused to the wild-type TIAM1 3'UTR. Expression of miR-10b reduced the activity of luciferase while a miR-10b seed mutant had no effect, indicating that miR-10b targets TIAM1 directly (Fig. 3D). As a control, I developed a second luciferase reporter with a single base pair mutation in the TIAM1 3'UTR, at the site corresponding to the miR-10b seed mutant. MiR-10b had no effect on the luciferase activity of this reporter, whereas the miR-10b seed mutant, a perfect match in the seed region, repressed the luciferase signal.

Next, I asked whether TIAM1 downregulation is responsible for inhibition of cell motility by miR-10b. To determine whether SUM-159PT cells are dependent on TIAM1 for cell motility, I diminished TIAM1 expression in these cells using a TIAM1 siRNA pool (Fig. 4A). Knockdown of TIAM1 resulted in a 40% decrease in both cell migration and cell invasion, similar to the change seen with *de novo* expression of miR-10b. Importantly, co-transfection of miR-10b and TIAM1 cDNA lacking the 3'UTR, and therefore the miR-10b target site, was able to rescue miR-10b-induced repression of cell motility (Fig. 4B).

TIAM1 is a guanine nucleotide exchange factor for Rac, a Rho-GTPase that regulates actin dynamics at the leading edge during cell movement. I hypothesized that miR-10b-induced downregulation of TIAM1 results in a

corresponding decrease in Rac activation, thereby impairing cell motility. Decreasing TIAM1 expression in SUM-159PT cells by siRNA resulted in a 60% decrease in Rac1 activation (Fig. 5A). Similarly, *de novo* expression of miR-10b in this cell line also repressed activation of Rac1 (Fig. 5B). Co-transfection of miR-10b and TIAM1 cDNA restored Rac1 activation to control levels (Fig. 5C).

Discussion

MiR-10b has been reported to be downregulated in breast cancer, however, it has also been postulated to promote cell motility and increase metastatic capabilities (Iorio et al., 2005; Ma et al., 2007). Recent evidence suggests that miR-10b is, in fact, not correlated with distant metastases, instead exhibiting an inverse correlation with tumor size, grade and vascular invasion (Gee et al., 2008). These discrepancies clearly show the need for more mechanistic studies regarding miR-10b and breast cancer. In the present study, I examined the role of miR-10b in various human breast cancer cell lines. Specifically, I utilized miR-10b null SUM-159PT and SUM-149PT cells in addition to miR-10b-expressing T47D cells. An interesting observation is that T47D cells, a differentiated epithelial strain derived from a pleural effusion of infiltrating ductal carcinoma (Keydar et al., 1979), are relatively non-motile *in vitro* and non-metastatic *in vivo*. SUM-159PT cells are an aggressive triple negative line (ER-/PR-/Her2-) derived from anaplastic carcinoma. These miR-10b null cells display a stellate morphology characteristic of a metastatic phenotype and develop

secondary tumors following orthotopic injection in nude mice (Flanagan et al., 1999). Similarly, SUM-149PT cells are derived from locally invasive inflammatory breast cancer, a particularly lethal form of breast cancer. These cells are postulated to undergo metastatic spread via a passive mechanism as tumor emboli, but are metastatic nonetheless (Hoffmeyer et al., 2005). This observation that miR-10b is expressed in an epithelial-like, non-metastatic cell line, but not in an invasive or aggressive metastatic cell line, is in direct contrast to previously published results that miR-10b is highly expressed only in metastatic breast cancer cells (Ma et al., 2007).

To assess miR-10b function in these cells, I first examined cell motility. Surprisingly, *de novo* expression of a miR-10b precursor in the highly motile SUM-159PT cells significantly repressed both cell migration and invasion, again in direct contrast to published results (Ma et al., 2007). A custom-designed miR-10b mutant, possessing a single base mutation in the critical seed region of miR-10b, showed little to no effect on cell motility, suggesting that the results obtained were specific to miR-10b. However, to ensure that I was not observing an artifact of the precursor miRNA, I went on to repeat these experiments using a retrovirus encoding the *miR-10b* gene, thereby allowing transcription and processing of miR-10b to occur endogenously. I was able to obtain the same retroviral plasmid used by Ma et al. (2007) for their experiments showing that miR-10b increases cell motility. Interestingly, I found that SUM-159PT cells transduced with the miR-10b retrovirus showed a significant reduction in both cell migration and invasion

in comparison to virus encoding the empty vector. These results support my earlier data obtained with the miR-10b precursor. However, to ensure that I was not observing a cell-type specific phenomenon, I transduced SUM-149PT cells, the same cells used by Ma et al. (2007), with the miR-10b retrovirus. These cells also demonstrated a significant decrease in both cell migration and invasion in comparison to empty vector controls. Unable to explain the striking discrepancy between my findings and the literature, I repeated the SUM-149PT cell assays under experimental conditions identical to the published protocol (Ma et al., 2007). My cell culture conditions were the same, and the cells themselves had been obtained from the same source. I modified my own protocol for the migration assays to use uncoated transwells for assessing migration towards media containing 10% serum over a 24 hour period and found that miR-10b again repressed cell migration. While I cannot rule out the possibility of acquired cell differences, the results obtained clearly indicate that miR-10b *suppresses* cell migration and invasion in breast carcinoma cells.

I was interested to conduct the reverse experiment and determine if inhibition of endogenous miR-10b would increase cell motility. For these experiments I utilized the relatively immotile, miR-10b-positive T47D cells and observed a striking increase in both cell migration and invasion in cells expressing miR-10b antisense. These results further indicate that miR-10b *suppresses* breast carcinoma cell migration and invasion, and refute published

data that miR-10b promotes breast cancer invasion and metastasis (Ma et al., 2007).

Review of putative miR-10b targets known to play a role in cell motility led us to TIAM1, a cytoplasmic protein that was originally identified through propagation of invasive cells (Habets et al., 1994). The results obtained show that TIAM1 protein levels are repressed by expression of miR-10b, and enhanced by inhibition of endogenous miR-10b. Luciferase assays confirmed that regulation of TIAM1 by miR-10b occurs via direct interaction at the TIAM1 3'UTR. The finding that miR-10b regulates TIAM1, a GEF for Rac, is a novel and exciting discovery. Further investigation showed that expression of a TIAM1 cDNA lacking the 3'UTR, and therefore immune to the effects of miR-10b, restored cell migration, suggesting that TIAM1 is the primary factor responsible for decreased cell motility in cells expressing miR-10b.

Rac1 functions downstream of TIAM1 to promote cytoskeletal rearrangement during cell migration (Ehler et al., 1997; Michiels et al., 1995; Stam et al., 1997) and my results confirm that TIAM1 expression is necessary for optimal Rac1 activation in breast carcinoma cells and identify an elegant mechanism in which miR-10b downregulates TIAM1 leading to decreased activation of Rac1 and a decrease in cell motility.

An important conclusion drawn from my data is that TIAM1-mediated Rac1 activation, migration, and invasion can be regulated by a specific miRNA. Although it is known that TIAM1 expression increases with breast cancer grade,

little is known about how this GEF is regulated in breast tumors. The ability of miR-10b to target TIAM1 provides one such mechanism, which is substantiated by the recent observation that miR-10b expression decreases as a function of grade in breast cancer (Gee et al., 2008).

My data contrast markedly with the data reported by Ma et al. (2007), who concluded that miR-10b promotes the migration and invasion of breast carcinoma cells by a mechanism that involves the HoxD10 induction of RhoC expression. Given that I used the same cell lines (e.g., SUM-149PT) and experimental conditions, it is difficult to reconcile this opposing conclusion of miR-10b function in breast cancer. My mechanistic data, however, support the recent observation that miR-10b expression in human breast tumors correlates inversely with a more invasive phenotype as indicated by tumor stage, grade and vascular invasion (Gee et al., 2008). I also question the purported role of miR-10b in inducing RhoC expression because our laboratory had reported previously that SUM-149PT cells, which were shown to lack miR-10b expression by both myself (Fig. 1) and Ma et al. (2007), express relatively high levels of RhoC (Simpson et al., 2004).

Materials and Methods

Cell lines: The SUM-159PT and SUM-149PT cell lines were obtained from Dr. Steve Ethier (University of Michigan) and cultured in HamsF12 supplemented with 5% fetal bovine serum, insulin (5 µg/ml, Sigma), hydrocortisone (1 µg/ml,

Sigma), and 1% penicillin-streptomycin. T47D and 293T cells were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). T47D cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.2 Units/ml bovine insulin, and 1% penicillin-streptomycin. 293T cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

RNA isolation and miRNA detection: Total RNA was isolated from cultured cells using the mirVana miRNA Isolation Kit (Ambion). Detection of the mature form of miRNAs was performed using the mirVana qRT-PCR miRNA Detection Kit and qRT-PCR Primer Sets, according to the manufacturer's protocols (Ambion). For qPCR, detection of the mature form of miRNAs was performed using TaqMan miRNA Reverse Transcription Kit and TaqMan Human Microarray Assays for miR-10b and miR-10b mutant (Ambion). U6 small nuclear RNA was used as an internal control.

Oligonucleotide transfection: Pre-miR miRNA Precursor Molecules (Ambion) are synthetic miRNA mimics designed for functional analyses and target site validation. Cells were transfected at 50% confluence with 20 nM of the following miRNA precursors using DharmaFECT 4 transfection reagent (Dharmacon): Pre-miR hsa-miR-10b Precursor, a custom-designed miR-10b seed mutant precursor with a single base pair substitution in the seed region of the mature strand, or a Pre-miR miRNA Precursor non-targeting Negative Control (Ambion). Seventy-two hours after transfection, cells were plated for migration and invasion assays,

or harvested for Rac activity assays. A custom designed 2'-O-methyl antisense oligonucleotide (Dharmacon) directed against mature miR-10b was used for loss-of-function analyses. Antisense oligonucleotides directed against luciferase were used for control. T47D cells were transfected with 20 nM of the antisense oligos as above. Nontargeting siRNA or siRNAs designed to target TIAM1 were SMART Pools from Dharmacon and cells were transfected with 20 nM of each pool. For TIAM1 rescue experiments, cells were transfected with 20-40 nM miRNA precursor and 0.6 pmol of a full length human TIAM1 full-length cDNA (Kathleen O'Connor, University of Texas) or empty vector control using DharmaFECT Duo transfection reagent (Dharmacon).

Migration and invasion assays: For migration assays, both the upper and lower surfaces of transwell chambers (8-µm pore, Costar) were coated overnight with collagen (15 µg/ml, Becton Dickinson) diluted in PBS. For invasion assays, the upper surface of the transwells was coated overnight with 0.5 µg Matrigel (Becton Dickinson). Cells were harvested at 80% confluence by trypsinization and resuspended in HamsF12 or RPMI-1640 containing 0.25% heat inactivated fatty acid-free BSA. The coated surfaces of the transwells were blocked with media containing 0.25% BSA for 30-60 minutes at 37^{o}C. Cells (2 x 10^{4} in a total volume of 100 µl) were loaded into the upper chamber and NIH-3T3-conditioned media or media containing 10% serum was added to the lower chamber. Assays proceeded for 4 hours for SUM-159PT cells, and 24 hours for SUM-149PT and T47D cells at 37^{o}C. Upon completion of the assay, the upper chamber was

swabbed to remove residual cells and fixed with methanol. Cells on the lower surface of the membrane were mounted in DAPI mounting media (Vector Laboratories) and the number of cells was determined for five independent fields with a 20X objective and fluorescence. Each experiment was performed in triplicate.

Constructs: The MDH1-PGK-GFP-premiR-10b and MDH1-PGK-GFP vectors were obtained from Addgene. 293T cells were transfected at 50% confluence by a Lipofectamine (Invitrogen) complex containing envelope plasmid (1.75 µg), packaging plasmid (3.25 µg), and MDH1-PGK-GFP-2.0 vector expressing pre-miR-10b or no insert in Optimem (Gibco). Two days following transfection the virus was harvested, clarified, and the supernatant was filtered through a 0.22-µm filter to be used immediately or stored at -80^{o}C. Recipient SUM-159PT and SUM-149PT cells were plated to reach 50% confluence after 24 hours, and virus was added to cells at a ratio of virus: fresh media containing Polybrene (8 µg/ml) of 1:1 and 1:2. For luciferase assay, a 60 bp region of the TIAM1 3'UTR containing the binding site for miR-10b was cloned into the pMIR-REPORT luciferase construct (Ambion). A second insert containing a single base pair mutation in the seed binding site, comparable to the miR-10b mutant, was cloned into the same construct to generate a luciferase construct with a mutated miR-10b binding site for control.

Immunoblotting: Whole cell lysates were prepared by lysis in ice-cold RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% NP-40, 1%

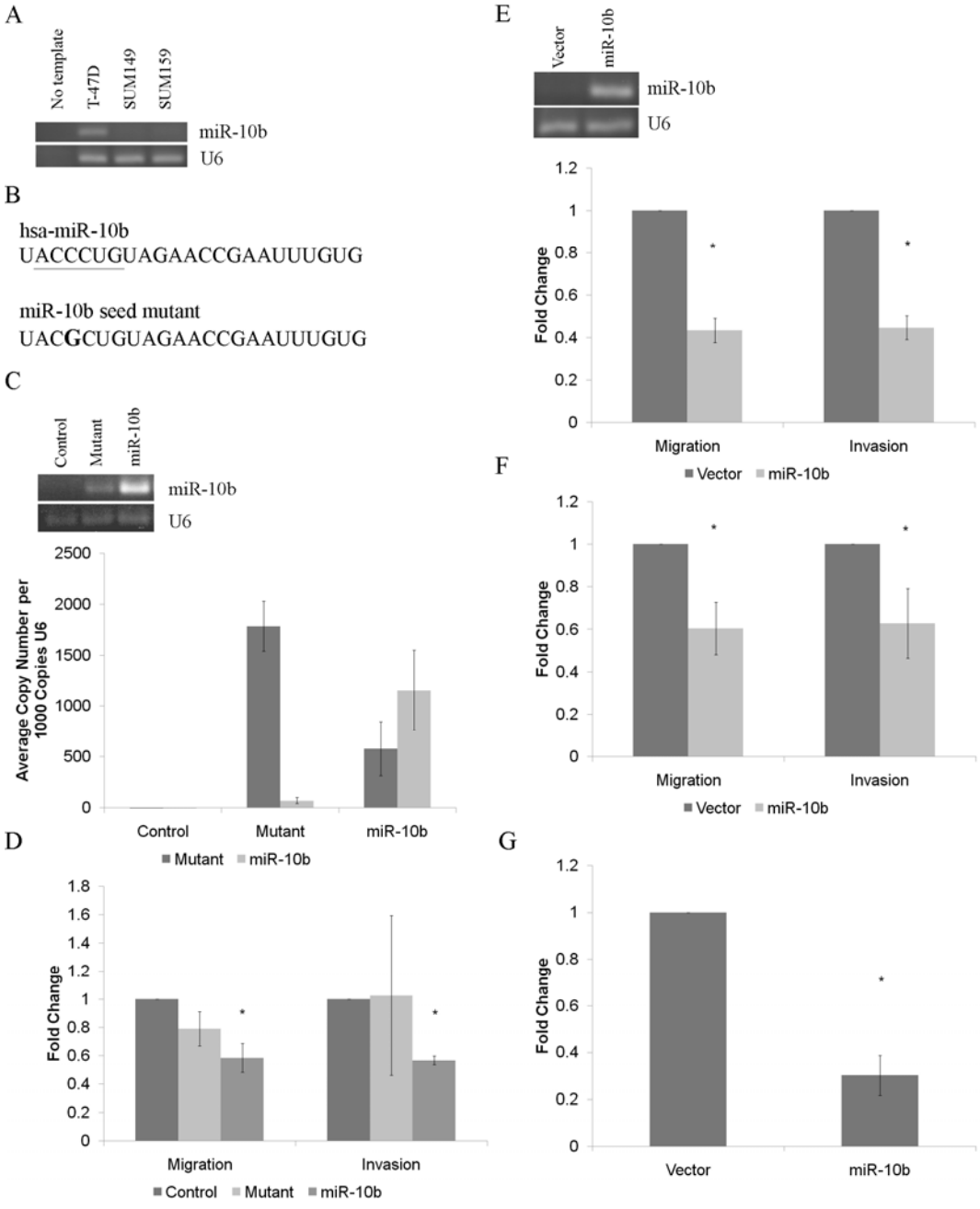
deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 µg/ml aprotinin, leupeptin, and pepstatin]. Lysates (50 µg) were separated by electrophoresis through 8 or 12% SDS-PAGE and transferred to 0.2 µm Nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked in 5% non-fat milk in TBS/Tween-20, blotted with the antibodies for TIAM1 (1:800, Santa Cruz), actin (1:5000, Sigma), or Rac1 (1:1000, Transduction Laboratories) overnight, followed by secondary peroxidase-conjugated anti-rabbit or anti-mouse antibodies, and detection was by enhanced chemiluminescence.

Luciferase Reporter Assay: Cells in 24-well plates at 50% confluence were co-transfected with a firefly luciferase reporter gene construct (200 ng – 0.5 µg) and 1 ng – 0.5 µg of Renilla-Luciferase construct (for normalization) using DharmaFECT Duo (Dharmacon). Cell extracts were prepared 24-48 hours after transfection and luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

Rac Activity Assays: Rac activity assays were based on established protocols (Benard et al., 1999; Sander et al., 1998a). The bacterially produced Rac/Cdc42 binding domain of Pak (PBD)-GST fusion protein was extracted and used to coat glutathione Sepharose beads (GE Healthcare). Serum starved cells were harvested by addition of ice-cold lysis buffer [50 mM Tris (pH 7.4), 100 mM NaCl, 1% NP-40, 10% glycerol, 2 mM $MgCl_2$, 2 mM PMSF, and 5 µg/ml each of aprotinin, leupeptin, and pepstatin]. Lysates were cleared by centrifugation and 0.1 total volume was removed to represent the total lysate control. GST-PBD-

coupled beads were added to the remaining lysates with 2 volumes of binding buffer [25 mM Tris (pH 7.5), 1 mM dithiothreitol, 40 mM NaCl, 30 mM $MgCl_2$, 0.5% Nonidet P-40] for 30 minutes on a rotating platform at 4^{o}C. Beads were washed three times in binding buffer and eluted in 2X Laemmli sample buffer. Aliquots of both total cell extracts and the eluents from the PBD beads were immunoblotted for Rac1.

Statistical analysis: Data are presented as the mean ± standard error of mean (SEM). The Student *t*-test was used to assess the significance of independent experiments. The criterion $p < 0.05$ was used to determine statistical significance.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T-47D
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B
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UACCCUGUAGAACCGAAUUUGUG
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Figure 1. MiR-10b suppresses breast carcinoma cell migration and invasion. A, RT-PCR of miR-10b in three different human breast cancer cell lines. **B**, Schematic showing the sequences of mature miR-10b and miR-10b seed mutant. The seed sequence of mature miR-10b is underlined. **C**, Upper panel, RT-PCR of miR-10b in SUM-159PT cells transfected with either non-targeting control miRNA, miR-10b mutant, or miR-10b precursor. Lower panel, quantitative Real-Time PCR of miR-10b and miR-10b mutant in SUM-159PT cells transfected with either non-targeting control miRNA, miR-10b mutant, or miR-10b precursor. Each primer set is capable of amplifying both miR-10b and miR-10b mutant, with higher fidelity for the exact sequence and lower fidelity for the non-identical sequence. **D**, Migration and Matrigel invasion assays of the transfected SUM-159PT cells. *, $P < 0.01$. **E**, Upper panel, RT-PCR of miR-10b in SUM-159PT cells infected with miR-10b-expressing or empty vector retrovirus. Lower panel, migration and Matrigel invasion assays of infected SUM-159PT cells. *, $P < 0.001$. **F**, Migration and Matrigel invasion assays of SUM-149PT cells infected with miR-10b-expressing or empty vector retrovirus. *, $P < 0.05$. **G**, Migration assay of infected SUM-149PT cells migrating towards 10% serum through uncoated transwells. *, $P < 0.001$. Data for migration and invasion assays represent means ± SEM from 3 independent experiments.

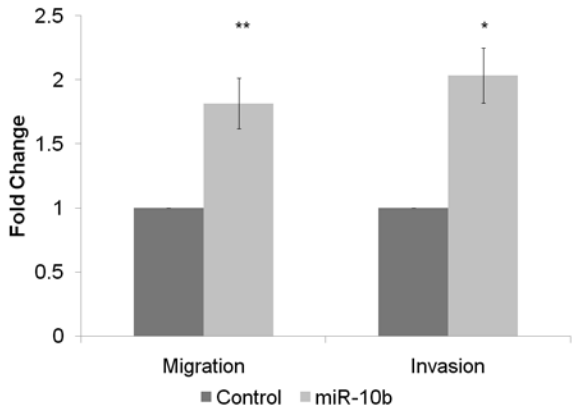

Figure 2. Inhibition of endogenous miR-10b increases T47D cell migration and invasion. Migration and Matrigel invasion assays of T47D cells transfected with miR-10b antisense oligonucleotides. Antisense oligonucleotides directed against luciferase were used for negative control. *, $P < 0.05$. **, $P < 0.005$. Data represent means ± SEM from 3 independent experiments.

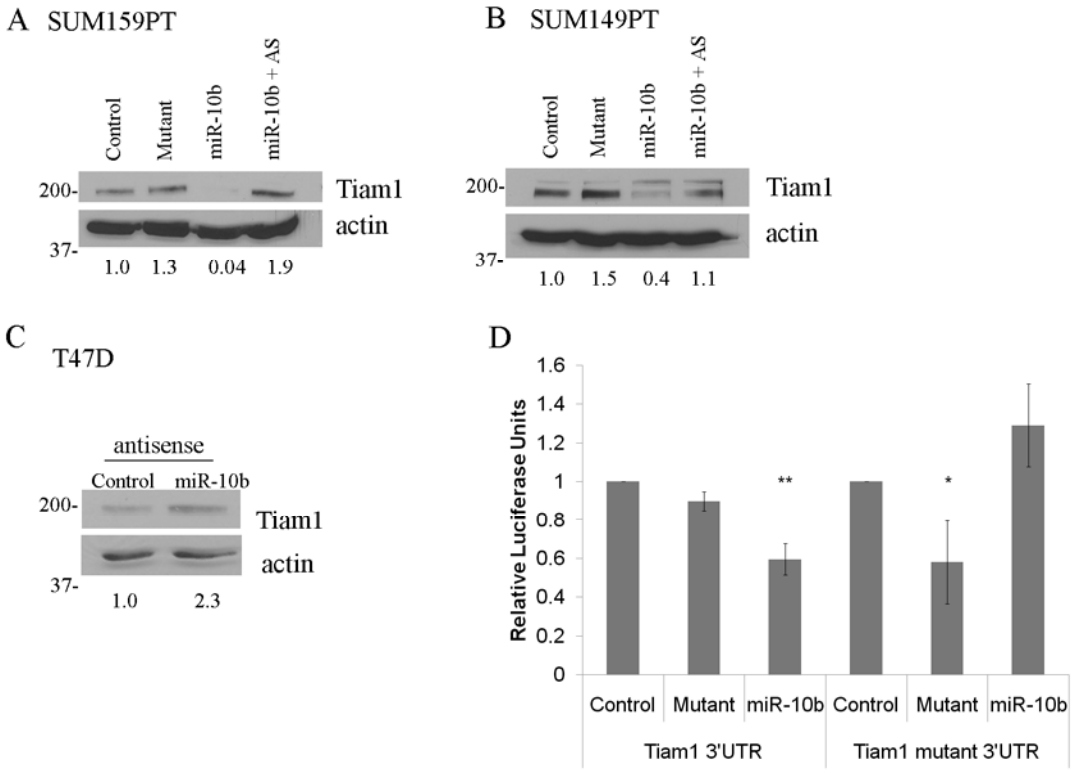

Figure 3. MiR-10b regulates expression of TIAM1. A, **B**, Immunoblot of TIAM1 expression in SUM-159PT (**A**) and SUM-149PT (**B**) cells transfected with either non-targeting control miRNA, miR-10b mutant, miR-10b precursor, or co-transfected with miR-10b precursor plus antisense miR-10b. **C**, Immunoblot of TIAM1 expression in T47D cells transfected with antisense oligonucleotides directed against luciferase (control) or miR-10b. The numbers under the blots represent densitometric analysis of the intensity of the TIAM1 bands relative to actin. **D**, Luciferase activity of a TIAM1 3'UTR or TIAM1 mutant 3'UTR reporter gene in SUM-159PT cells transfected with either miR-10b, miR-10b mutant, or non-targeting control miRNA. *, $P < 0.05$. **, $P < 0.005$. Data represent means ± SEM from 3 independent experiments.

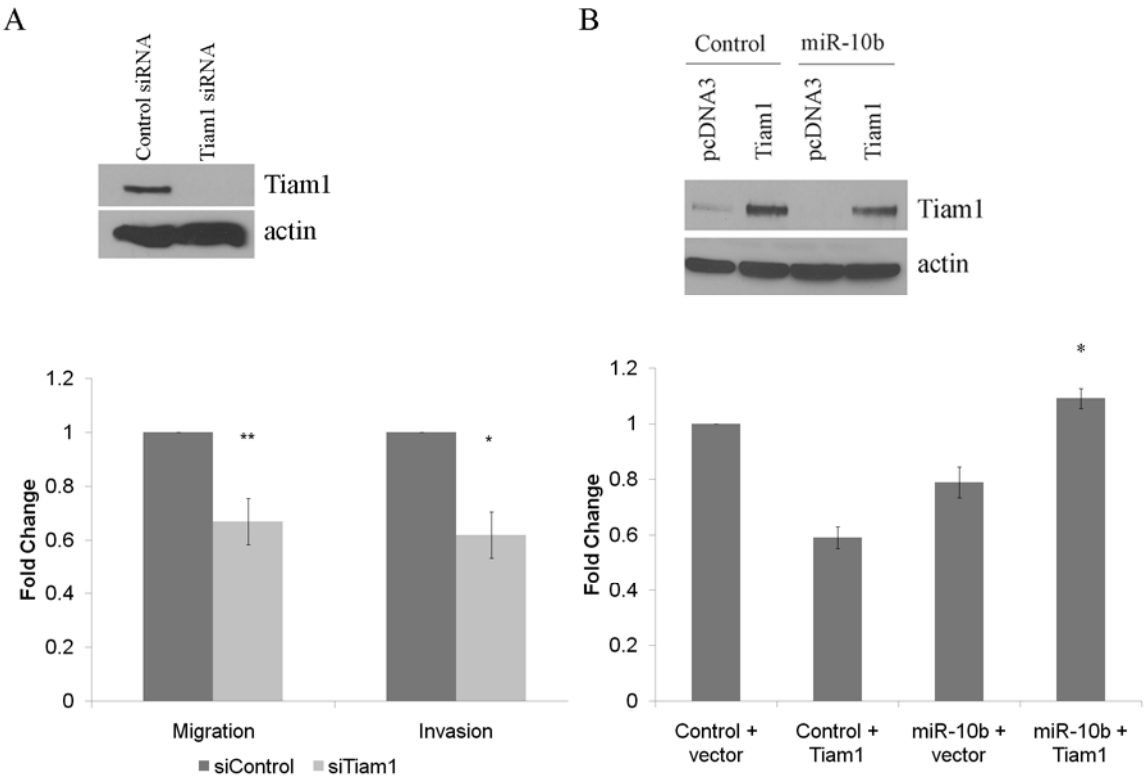

Figure 4. Exogenous expression of TIAM1 rescues cell migration. A, Immunoblot of TIAM1 expression (upper panel) and migration and Matrigel invasion assays (lower panel) of SUM-159PT cells transfected with either a TIAM1 siRNA pool or a control siRNA pool. *, $P < 0.01$. **, $P < 0.001$. Data for migration and invasion assays represent means ± SEM from 3 independent experiments. **B**, Immunoblot (upper panel) and migration assay (lower panel) of SUM-159PT cells co-transfected with miR-10b or non-targeting control miRNA, and either a TIAM1 cDNA lacking the 3'UTR or empty vector. *, $P < 0.001$.

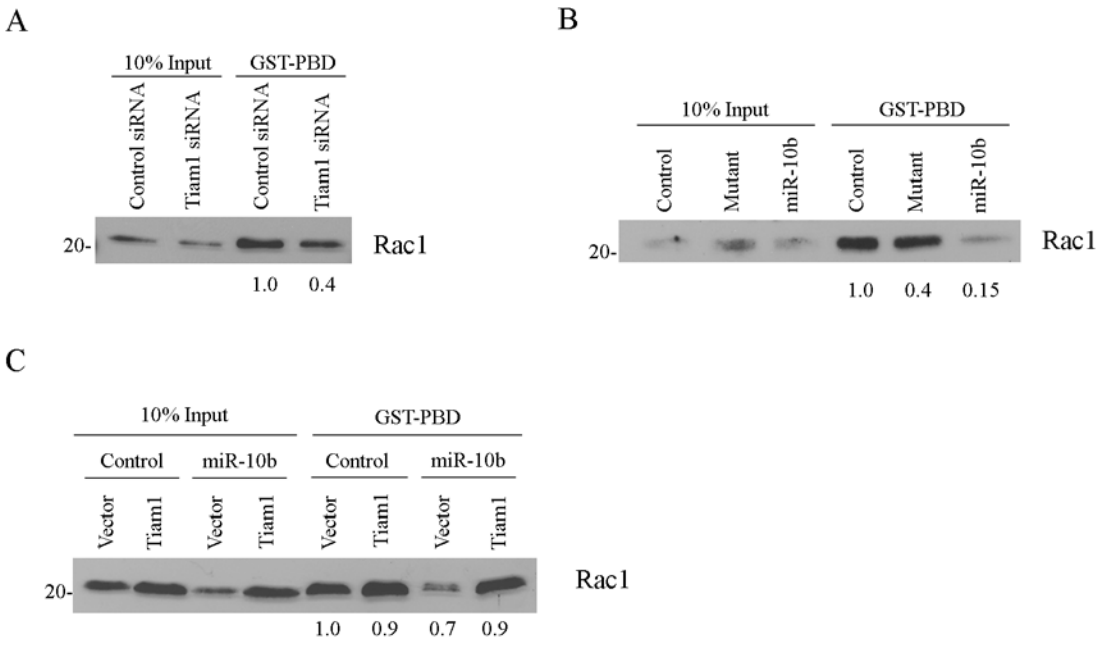

Figure 5. MiR-10b represses TIAM1-dependent activation of RAC1. SUM-159PT cells were transfected with either a TIAM1 siRNA pool or a control siRNA pool (**A**), with either non-targeting control miRNA, miR-10b mutant, or miR-10b precursor (**B**), or with non-targeting control miRNA or miR-10b precursor co-transfected with a TIAM1 cDNA lacking the 3'UTR or empty vector (**C**). Cell extracts were analyzed for Rac1 activation using the PBD assay as described in Materials and Methods. The numbers under the blots represent densitometric analysis of the intensity of the Rac1 bands.

CHAPTER III

VEGF RECEPTOR FLT-1 REGULATION BY miR-10b CONTRIBUTES TO BREAST CANCER CELL MOTILITY

Introduction

VEGF produced locally by either tumor or stromal cells, in addition to its role in angiogenesis, engages VEGF receptors on tumor cells and initiates a signaling response that facilitates survival (Bachelder et al., 2002; Bates et al., 2003; Das et al., 2005; Fragoso et al., 2006; Lee et al., 2007; Lipscomb et al., 2005; Weigand et al., 2005). The VEGF/PlGF receptor FLT-1 is upregulated in many common tumors, including breast, and has been found to mediate VEGF autocrine signaling loops essential for survival in colon carcinoma cells (Bates et al., 2003) and in neuroblastoma cells exposed to hypoxia (Das et al., 2005). In breast carcinoma, FLT-1 has been identified as a key component of a ‘poor prognosis gene signature’ that is strongly predictive of a short interval to distant metastases (van 't Veer et al., 2002) and FLT-1 expression in primary tumors has been correlated with a high risk of metastasis and relapse (Ghosh et al., 2008; Meunier-Carpentier et al., 2005; Mylona et al., 2007). Furthermore, anti-FLT-1 antibody or FLT-1 specific inhibitory peptides are reported to suppress tumor growth and metastasis in various tumor models, including breast (Bae et al.,

2005; Taylor and Goldenberg, 2007; Wu et al., 2006). Based on these findings, I hypothesize that FLT-1 may contribute to the migration and invasion of breast tumor cells, thereby promoting an aggressive phenotype.

MiR-10b is one of 5 miRNAs whose expression has been reported to be significantly downregulated in breast cancer (Iorio et al., 2005). Furthermore, miR-10b expression correlated inversely and significantly with tumor size, grade and vascular invasion in a cohort of patients with early breast cancer (Gee et al., 2008). These data infer that miR-10b impedes specific functions associated with breast cancer progression. In support of this notion, I have shown that miR-10b suppresses migration and invasion of breast carcinoma cells via downregulation of GEF TIAM1 and inhibition of TIAM1 dependent Rac activation (Chapter 2). Interestingly, FLT-1 is also a putative target of miR-10b, but the function of FLT-1 in breast cancer remains largely unknown.

In this study, I present my analysis of FLT-1 function in breast carcinoma cells, which revealed that it is a valid target of miR-10b and that it functions to suppress both cell migration and invasion.

Results

To assess the ability of miR-10b to regulate FLT-1 expression in breast cancer, I used a miR-10b precursor for *de novo* expression in a miR-10b-null, highly invasive breast carcinoma cell line, SUM-159PT (Flanagan et al., 1999;

Ma et al., 2007). Transient expression of miR-10b in SUM-159PT cells resulted in expression of mature miR-10b, as assessed by RT-PCR (Fig. 1A). I observed a dramatic downregulation of FLT-1 protein levels in cells expressing miR-10b as compared to non-targeting control miRNA (Fig. 1B).

The search program Targetscan revealed that VEGF co-receptor neuropilin 2 (NRP2) is also a putative target of miR-10b. NRP2 has recently been implicated as a positive factor in cancer metastasis (Caunt et al., 2008). To assess the validity of this target, miR-10b was expressed in MDA-MB-435 cells, which express both endogenous miR-10b and NRP2. Transient overexpression of miR-10b caused a significant suppression of FLT-1 protein expression, but did not suppress NRP2 as compared to non-targeting control miRNA (Fig. 1C).

Since both *de novo* expression and overexpression of miR-10b suppressed expression of FLT-1, I next asked whether inhibition of endogenous miR-10b would increase FLT-1 expression. For this purpose, I expressed a miR-10b antisense oligonucleotide or control antisense directed against luciferase in miR-10b-positive T47D breast cancer cells. Immunoblot analysis revealed a significant increase of FLT-1 protein in cells expressing miR-10b antisense (Fig. 1D).

FLT-1 expression in breast cancer has been previously correlated with poor prognosis and a high risk of metastasis and relapse, but its function is poorly understood. To examine the function of FLT-1 in breast cancer, I turned to FLT-1 RNAi to suppress FLT-1 expression for loss-of-function studies. I found

that FLT-1 was very difficult to knockdown in breast cancer cell lines. Commercially available siRNA pools, shRNA viral vectors, and published siRNA sequences reported to knockdown FLT-1 in endothelial cells (Kou et al., 2005) all failed to repress FLT-1 expression in my hands (data not shown). Therefore, I designed a unique siRNA to the FLT-1 3'UTR, proximal to the miR-10b target site. This siRNA successfully and significantly repressed expression of FLT-1 in SUM-159PT cells (Fig. 2A).

Since I have previously shown that miR-10b suppresses breast cancer cell motility, and FLT-1 is extensively implicated in breast cancer progression, I hypothesized that FLT-1 contributes to cell migration and invasion in breast cancer. To determine the best chemoattractant for cell motility assays, serum-starved cells were placed in the upper chamber of transwells coated with 15 µg/ml collagen and allowed to migrate towards serum-free medium (SFM), NIH-3T3 conditioned medium, or physiologic concentrations of PlGF, VEGF, or VEGF plus heparin. I observed robust migration towards NIH-3T3-conditioned medium, however, the cells did not exhibit enhanced migration towards either PlGF or VEGF in comparison to SFM (Fig. 2B, 2C). Following transfection with FLT-1 siRNA, cells were seeded into the upper chamber of transwells coated with 15 µg/ml collagen and 0.5 µg matrigel to assess the role of FLT-1 in migration and invasion, respectively, toward NIH-3T3-conditioned medium. Knockdown of FLT-1 resulted in approximately 30% reduction in cell migration and greater than 50% reduction in cell invasion (Fig. 2D).

FLT-1 is receptor tyrosine kinase for VEGF and has been reported to promote cancer metastasis via its kinase activity (Shibuya, 2006). Furthermore, SUM-159PT cells engage in VEGF autocrine survival signaling (Lipscomb et al., 2005). Therefore, I next examined FLT-1 auto-phosphorylation. Cells were serum-starved and treated with or without VEGF or serum. Auto-phosphorylation was assessed by FLT-1 immunoprecipitation followed by phosphotyrosine immunoblot. I did not observe phosphorylation of FLT-1 under any of the conditions tested (data not shown).

Since I failed to detect tyrosine phosphorylation of FLT-1, I next asked whether FLT-1 might be mutated in breast cancer, and specifically in the SUM-159PT cells. A single amino acid substitution in the activation loop was recently reported to define the decoy characteristic of FLT-1 in endothelial cells (Meyer et al., 2006). To examine the sequence of FLT-1 in SUM-159PT cells for a similar mutation, I isolated genomic DNA from this cell line and sequenced all 30 exons of the FLT-1 gene using Transgenomic Surveyor/WAVE sequencing technology. Heterozygous analysis by denaturing HPLC revealed a C-T single-nucleotide polymorphism in the intron region preceeding exon 13 (nucleotides 1908-2004) (Fig. 3). Homozygous analysis comparing FLT-1 in SUM-159PT breast cancer cells to wild-type FLT-1 from human blood showed identical sequences with no mutations present.

Discussion

Extensive studies in patient populations have identified FLT-1 as a key factor in poor prognosis breast cancer (Meunier-Carpentier et al., 2005; van 't Veer et al., 2002). Specifically, increased expression of FLT-1 in primary breast cancer correlates with a high risk of metastasis and relapse (Ghosh et al., 2008; Meunier-Carpentier et al., 2005; Mylona et al., 2007). Furthermore, treatment with anti-FLT-1 antibody or FLT-1 inhibitory peptides suppresses breast cancer growth and metastasis (Bae et al., 2005; Luttun et al., 2002; Taylor and Goldenberg, 2007; Wu et al., 2006). However, only one study to date has examined FLT-1 function in breast cancer, reporting that FLT-1 is involved in an internal autocrine survival signaling pathway (Lee et al., 2007). Based on these findings, FLT-1 plays a key role in breast cancer progression, but further study is necessary to examine FLT-1 function as a potential therapeutic target.

I have previously identified miR-10b as a suppressor of cell motility in breast cancer via TIAM1-mediated Rac1 activation. Interestingly, FLT-1 is a potential target of miR-10b as well, identified by another group via TargetScan following the finding that miR-10b is significantly downregulated in breast cancer (Iorio et al., 2005). I hypothesized that FLT-1 is regulated by miR-10b and contributes to breast cancer cell motility. In the present study, I show that FLT-1 is downregulated following *de novo* expression of miR-10b in breast cancer cells, indicating that FLT-1 is, in fact, a target of miR-10b. Interestingly, during the course of this work the sequence of FLT-1 was annotated in the NCBI database,

eliminating the miR-10b target site from the 3'UTR. As a result, TargetScan no longer identifies FLT-1 as a target of miR-10b. Another commonly used computer algorithm, PicTar, does identify FLT-1 as a putative miR-10b target. The current predictions by TargetScan and PicTar have a high degree of overlap because they both require stringent seed pairing between a miRNA and its target mRNA. However, differences may arise due to alignment artifacts, the use of different UTR databases, the use of different miRNA sequences, or the different prediction algorithms themselves (Bartel, 2009).

Another putative target of miR-10b that is reported to play a role in cancer metastasis is VEGF co-receptor NRP2 (Caunt et al., 2008). SUM-159PT cells do not express NRP2, so I assessed the validity of this target in MDA-MB-435 cells. Although these cells express endogenous miR-10b, I found that transient overexpression of miR-10b caused significant downregulation of FLT-1 protein, but did not decrease NRP2 protein levels, suggesting that NRP2 may not be a valid target of miR-10b. In fact, TargetScan no longer identifies NRP2 as a miR-10b target either. Similar to FLT-1, NRP2 is predicted to be a miR-10b target by PicTar, indicating that these predictions are subject to error and that experimental validation of miRNA targets is critical.

I next examined whether inhibition of endogenous miR-10b increases FLT-1 protein expression. Inhibition of endogenous miR-10b in miR-10b-positive T47D cells would be expected to increase expression of FLT-1 protein by releasing miR-10b-induced downregulation. Indeed, I observed a significant

increase in FLT-1 protein by immunoblot, further indicating FLT-1 is a target of miR-10b in breast cancer.

A recent study suggests that FLT-1 acts as part of an internal autocrine VEGF signaling pathway in breast cancer, thereby facilitating cell survival (Lee et al., 2007). To further examine the function of FLT-1 in breast cancer cells, I turned to RNAi. FLT-1 expression was easily suppressed by both expression and overexpression of miR-10b in our models. However, numerous FLT-1 siRNAs failed to knockdown FLT-1 expression. MiRNA sites in the mRNA 3'UTR, which interact with miRNA-loaded RNA-induced silencing complex (miRISC) for posttranscriptional gene regulation, provide alternative potentially accessible sites for siRNA (Wu et al., 2008). Given the success of miR-10b in suppressing FLT-1 via the 3'UTR, I designed a unique siRNA to the 3'UTR in close proximity to the miR-10b target site. Our siRNA is not predicted to target any other known mRNAs by BLAST. A recent publication investigated "difficult-to-silence" target mRNAs for which no functionally validated siRNAs are available, finding that siRNAs directed against the 3'UTR generally caused higher knockdown than previously designed siRNAs for these targets. In general, knockdown by siRNAs targeting the miRNA seed region was specific for the target mRNA, and siRNAs targeting 1 nt upstream of miRNA seed region were similarly potent (Bergauer et al., 2009). These findings indicate that siRNA to the 3'UTR is a valid and successful approach for "difficult-to-silence" target mRNAs such as FLT-1.

For FLT-1 loss-of-function studies I first looked to cell motility. I have previously shown that miR-10b suppresses breast cancer cell migration and invasion, and I hypothesized that FLT-1 may be contributing to this process. FLT-1 has been shown to promote the migration of monocytes and macrophages, as well as migration and invasion of pancreatic and colon cancer cells (Fan et al., 2005; Lesslie et al., 2006; Wey et al., 2005), suggesting that FLT-1 may play a universal role in cell motility. In fact, I observed a 0.5-fold and greater than 2-fold decrease in breast carcinoma cell migration and invasion, respectively, suggesting that FLT-1 promotes cell migration and, to a greater extent, invasion in breast cancer.

To begin to examine the mechanism by which FLT-1 promotes breast cancer cell motility, I assessed FLT-1 auto-phosphorylation. FLT-1 promotes cancer metastasis via its kinase activity (Shibuya, 2006). I hypothesized that an increase in FLT-1 auto-phosphorylation activates downstream pathways which promote cell migration and invasion. Surprisingly, I found no evidence of FLT-1 tyrosine auto-phosphorylation. Since PlGF and VEGF stimulation failed to induce chemotaxis or tyrosine phosphorylation, it is possible that the internal autocrine signaling pathway previously identified (Lee et al., 2007) may account for ligand-independent migration in these cells. Additionally, expression of FLT-1 may promote a basal level of signaling (perhaps by an autocrine mechanism) that contributes to cell motility. Further investigation to examine the localization of FLT-1 in these cells is warranted.

Given the failure of previously validated siRNAs and the lack of tyrosine auto-phosphorylation in breast cancer, I asked whether FLT-1 is mutated in breast cancer. A mutation in the FLT-1 coding sequence has the potential to not only disrupt siRNA binding, but also to significantly alter protein function. Interestingly, a single amino acid substitution in the activation loop of FLT-1 has been reported to define the decoy characteristic of FLT-1 in endothelial cells (Meyer et al., 2006). I hypothesized that FLT-1 might have a similar mutation in breast cancer. However, extensive sequencing analysis revealed that FLT-1 has no mutations in the SUM-159PT breast cancer cell line. Homozygous analysis comparing FLT-1 in breast cancer to wild-type FLT-1 in human blood showed no differences, while heterozygous analysis of the two FLT-1 alleles in SUM-159PT cells showed only a single nucleotide polymorphism located in an intron region and therefore likely to be silent. Collectively, these results suggest that FLT-1 is not mutated in SUM-159PT breast cancer cells.

My data show, for the first time, that the VEGF receptor FLT-1 can be regulated by a specific miRNA. Although it is known that FLT-1 expression correlates with a poor prognosis in breast cancer, little is known about its function or how it is regulated in breast tumors. The ability of miR-10b to target FLT-1 provides one mechanism for regulation of FLT-1 expression, which is substantiated by the recent finding that miR-10b expression decreases as a function of breast cancer grade (Gee et al., 2008).

Importantly, I have identified a novel function for FLT-1 in promoting breast cancer cell migration and invasion. These data are in agreement with previous findings that FLT-1 expression correlates with a high risk of metastasis and that inhibition of FLT-1 by anti-FLT-1 antibody or specific inhibitory peptides suppresses breast cancer growth and metastasis (Bae et al., 2005; Taylor and Goldenberg, 2007; Wu et al., 2006). Although further studies are necessary to examine the downstream pathways of FLT-1 in breast cancer cell motility, these data provide evidence that FLT-1 plays a key role in promoting breast cancer.

Materials and Methods

Cell lines: The SUM159PT cell line was obtained from Dr. Steve Ethier (University of Michigan) and cultured in HamsF12 supplemented with 5% fetal bovine serum, insulin (5 µg/ml, Sigma), hydrocortisone (1 µg/ml, Sigma), and 1% penicillin-streptomycin. MDA-MB-435 and T47D cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). MDA-MB-435 cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. T47D cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 0.2 Units/ml bovine insulin, and 1% penicillin-streptomycin.

RNA isolation and miRNA detection: Total RNA was isolated from cultured cells using the mirVana miRNA Isolation Kit (Ambion). Detection of the mature form of miRNAs was performed using the mirVana qRT-PCR miRNA Detection

Kit and qRT-PCR Primer Sets, according to the manufacturer's protocols (Ambion). U6 small nuclear RNA was used as an internal control.

Oligonucleotide transfection: Pre-miR miRNA Precursor Molecules (Ambion) are synthetic miRNA mimics designed for functional analyses and target site validation. Cells were transfected at 50% confluence with 20 nM of Pre-miR hsa-miR-10b Precursor or a Pre-miR miRNA Precursor non-targeting Negative Control using DharmaFECT 4 transfection reagent (Dharmacon). Seventy-two hours after transfection, cells were plated for migration and invasion assays. A locked nucleic acid miR-10b antisense oligonucleotide and 2'-O-methyl antisense oligonucleotide (Dharmacon) targeting mature miR-10b were used for loss-of-function analyses. Antisense oligonucleotides targeting luciferase were used for negative control. T47D cells were transfected with 20 nM of antisense oligo as above. FLT-1 siRNA was designed to target the FLT-1 3'UTR (Sense strand: 5'-GCCUACUCUUCAGGGUCUAGC-3', Antisense strand: 5'-UAGACCUGAAGAGUAGGCGC-3'). Control siRNA is a non-targeting negative control siRNA (Ambion). Cells were transfected with 20 nM of each siRNA.

Migration and invasion assays: For migration assays, both the upper and lower surfaces of transwell chambers (8-µm pore, Costar) were coated overnight with collagen (15 µg/ml, Becton Dickinson) diluted in PBS. For invasion assay, the upper surface of the transwells were coated overnight with 0.5 µg Matrigel (Becton Dickinson). Cells were harvested at 80% confluence by trypsinization and resuspended in HamsF12 containing 0.25% heat inactivated fatty acid-free

BSA. The coated surfaces of the transwells were blocked with media containing 0.25% BSA for 30-60 minutes at 37^{o}C. Cells (2 x 10^{4} in a total volume of 100 µl) were loaded into the upper chamber and NIH-3T3-conditioned media or media containing 5 ng/ml PlGF, 10 ng/ml PlGF, 50 ng/ml VEGF, or 50 ng/ml VEGF plus heparin was added to the lower chamber. Assays proceeded for 4 hours at 37^{o}C. At completion of the assay, the upper chamber was swabbed to remove residual cells and fixed with methanol. Cells on the lower surface of the membrane were mounted in DAPI mounting media (Vector Laboratories) and the number of cells was determined for five independent fields in triplicate with a 20X objective and fluorescence.

Immunoblotting: Whole cell lysates were prepared by lysis in ice-cold RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 ug/ml aprotinin, leupeptin, and pepstatin]. Lysates (50 µg) were separated by electrophoresis through 8% SDS-PAGE and transferred to 0.2 µm Nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked in 5% non-fat milk in TBS/Tween-20, blotted with the antibodies for FLT-1 (1:500, R&D Systems) or actin (1:5000, Sigma) overnight, followed by secondary peroxidase-conjugated anti-goat or anti-rabbit antibodies, and detection was by enhanced chemiluminescence.

Statistical analysis: Data are presented as the mean ± standard error of mean (SEM). The Student *t*-test was used to assess the significance of independent experiments. The criterion $p < 0.05$ was used to determine statistical significance.

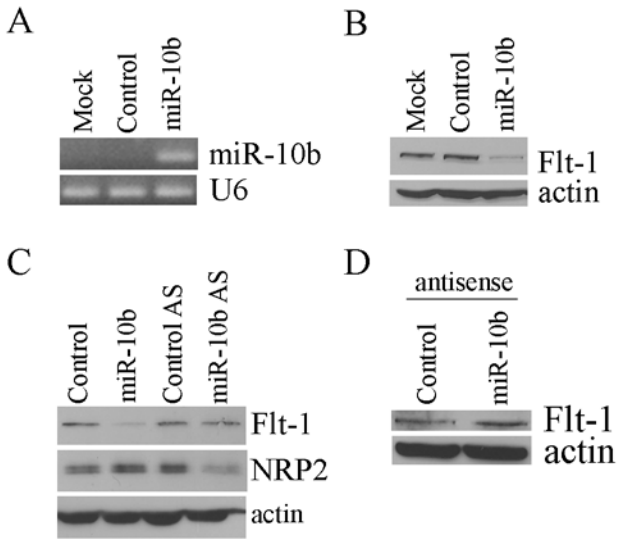

Figure 1. MiR-10b suppresses expression of VEGF receptor FLT-1. **A,B,** RT-PCR of miR-10b (**A**) and immunoblot of FLT-1 (**B**) in SUM-159PT cells transfected with either miR-10b precursor, non-targeting control miRNA, or transfection reagent alone (mock). **C**, Immunoblot of FLT-1 and NRP2 expression in MDA-MB-435 cells transfected with either miR-10b precursor, non-targeting control miRNA, or transfection reagent alone (mock). **D**, Immunoblot of FLT-1 expression in T47D cells transfected with either miR-10b antisense or control antisense.

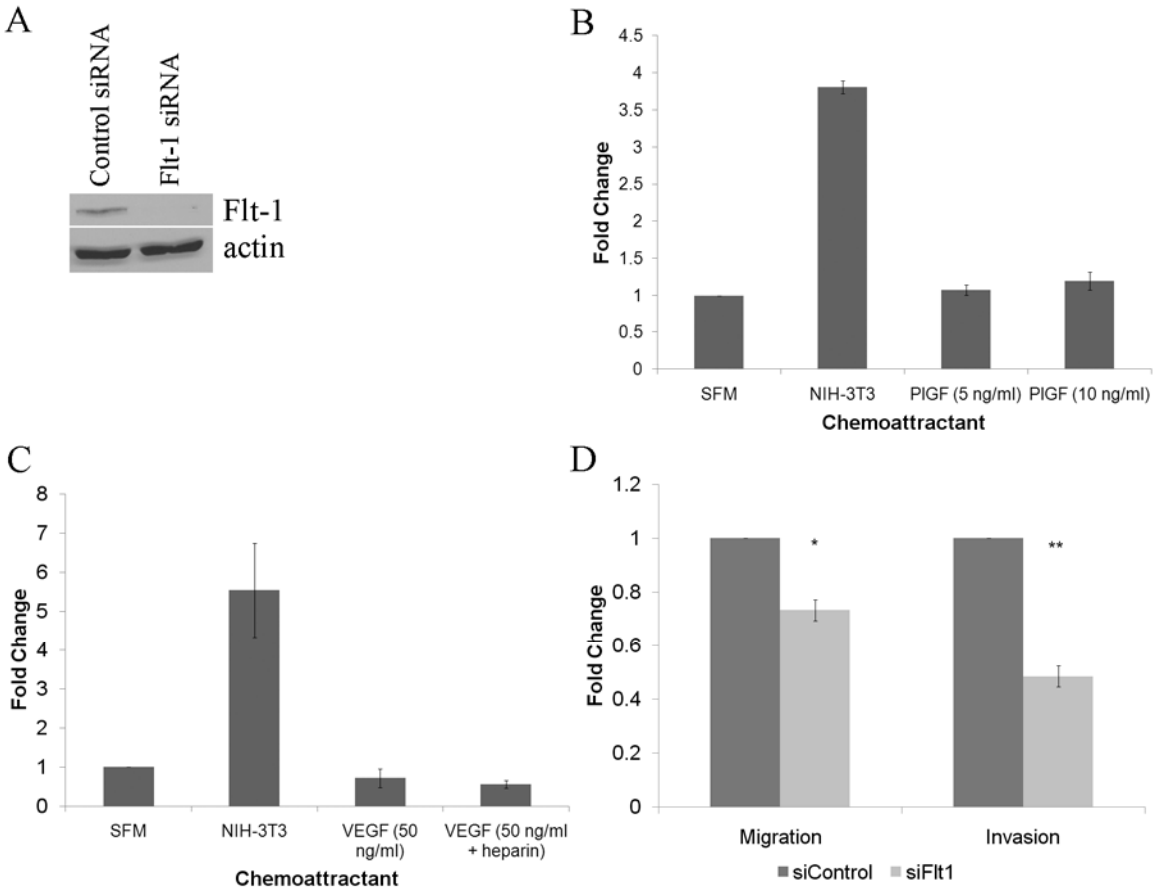

Figure 2. FLT-1 promotes breast cancer cell migration and invasion. A, Immunoblot of FLT-1 in SUM-159PT cells transfected with FLT-1 siRNA directed to the FLT-1 3'UTR or non-targeting control siRNA. **B,C,** Migration assays of SUM-159PT cells towards various chemoattractants, including SFM, NIH-3T3-conditioned media, PlGF (**B**), and VEGF (**C**). **D,** Migration and Matrigel invasion assays of SUM-159PT cells transfected with FLT-1 siRNA or non-targeting control siRNA. *, $P < 0.05$. **, $P < 0.0001$. Data for migration and invasion assays represent means ± SEM from 3 independent experiments.

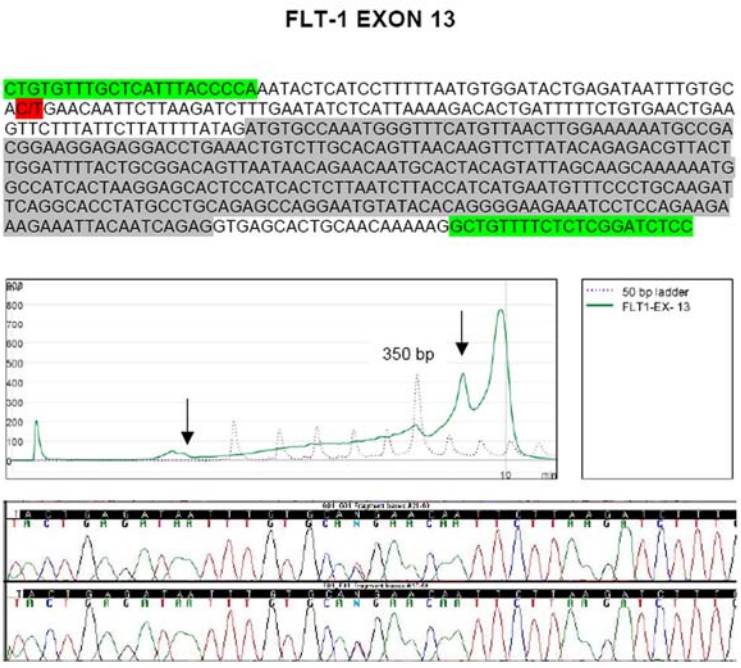

Figure 3. FLT-1 contains a C-T single nucleotide polymorphism in the intron region preceeding exon 13. Heterozygous analysis of FLT-1 in SUM-159PT cells by denaturing HPLC using Transgenomic Surveyor/WAVE sequencing technology.

CHAPTER IV

GENERAL DISCUSSION

The acquisition of a motile and invasive phenotype is an important step in the development of tumors and tumor metastases (Hanahan and Weinberg, 2000), requiring the abrogation of cell-cell contacts, remodeling of the ECM and cell-matrix interactions, and movement of the cell mediated by the actin cytoskeleton. To date, the mechanisms of regulation of these processes in breast cancer are poorly understood. An understanding of the ability of a carcinoma cell to acquire the migratory and invasive capabilities that lead to metastatic disease is a key area for identifying mechanisms of cancer progression and preventing metastasis in a clinical setting. MiRNAs have the ability to regulate a large number of genes simultaneously and thus provide an attractive model for global gene regulation during cancer progression (Tavazoie et al., 2008).

Recent evidence has shown that deregulation of miRNA expression correlates with various human cancers, including breast (Calin and Croce, 2006; Iorio et al., 2005; Lu et al., 2005). Furthermore, several miRNAs, including miR-10b, have been found to play a role in cancer cell motility and metastasis (Burk et al., 2008; Huang et al., 2008; Korpal et al., 2008; Tavazoie et al., 2008; Zhu et al., 2008). In recent years, several studies have addressed miR-10b in breast

cancer with conflicting results. The first study found that miR-10b is downregulated in breast cancer and identified miR-10b as one of 15 miRNAs in a miRNA signature able to correctly predict the nature of a tissue sample (i.e. normal vs. tumor) (Iorio et al., 2005). A later study supported this notion, with the finding that miR-10b correlates inversely with breast tumor size and grade (Gee et al., 2008). However, the third study was in disagreement, reporting that miR-10b acts as a pro-metastatic agent in breast cancer via regulation of HoxD10 and RhoC (Ma et al., 2007). In direct contradiction to Gee et al. (2008), miR-10b was found to correlate significantly with breast cancer cell progression (Ma et al., 2007). Taken together, the accumulated data suggest that miR-10b may play a role in breast cancer progression, but fail to reach a consensus on the role of miR-10b in breast cancer. Thus, I sought to examine the function of miR-10b in breast cancer cells. In this thesis, I have established the role of miR-10b in suppressing breast cancer cell migration and invasion via regulation of GEF TIAM1 and decreased activation of the Rho GTPase Rac1. I have also provided evidence that miR-10b regulates expression of the VEGF receptor FLT-1 and identified a novel role for FLT-1 in promoting breast cancer cell motility. Taken together our data suggest that miR-10b provides a novel mechanism for suppressing breast cancer cell motility via downregulation of key proteins.

MiR-10b Regulation of Cell Migration

As described in Chapter 2, I have demonstrated that miR-10b suppresses both cell migration and invasion using two different aggressive human breast carcinoma cell lines. Similarly, inhibition of endogenous miR-10b in a relatively non-motile breast carcinoma cell line increased cell migration and invasion. These findings indicate that miR-10b functions as a negative regulator of cell migration and invasion in breast cancer.

MiRNAs have the potential to regulate a number of mRNAs simultaneously. However, disrupting the miRNA regulation of a single target can have significant phenotypic effects (Bartel, 2007). Thus, it is likely that a single miR-10b target plays a seminal role in breast cancer cell motility. Examination of common miRNA target prediction computer algorithms revealed a multitude of targets, including two targets – TIAM1 and FLT-1 – previously implicated in cell motility. I have found that miR-10b targets both TIAM1 (Chapter 2) and FLT-1 (Chapter 3) in breast cancer cell lines.

Interestingly, miR-10b decreases activation of the Rho GTPase Rac1 through downregulation of TIAM1. Rac1 functions as a molecular switch, promoting cell migration via regulation of the actin cytoskeleton when bound to GTP. These results show, for the first time, that Rac1 activation can be regulated by a specific miRNA, thereby providing a new dimension to regulation of cell motility.

Implications for TIAM1 and Rac1

TIAM1 is a GEF for the Rho GTPases Rac, Rho, and Cdc42. TIAM1 plays a dual role in cell motility, promoting cell adhesion in epithelial cell types while promoting migration and invasion in other cell types via activation of Rac1 (Minard et al., 2004). In breast cancer, TIAM1 increases the migratory and invasive phenotypes of breast cancer cell lines (Minard et al., 2004). Furthermore, increased expression of TIAM1 is closely correlated to the invasiveness of breast tumor cells and the degree of progression of breast tumors (Adam et al., 2001). However, the regulation of TIAM1 expression in breast cancer has not been investigated. As discussed in Chapter 2, miR-10b directly downregulates expression of TIAM1 by binding to its 3'UTR. SUM-159PT breast cancer cells are dependent on TIAM1 expression for optimal activation of Rac1, an important factor in cell motility. Downregulation of TIAM1 in these cells results in decreased activation of Rac1. Importantly, cell motility and Rac1 activation can be rescued by expression of a TIAM1 cDNA lacking the 3'UTR, and therefore the miR-10b target site, suggesting that TIAM1 plays a key role in miR-10b-induced suppression of cell motility. These data show, for the first time, that TIAM1-mediated Rac1 activation and migration/invasion can be regulated by a specific miRNA. Furthermore, our findings provide a novel mechanism for the regulation of TIAM1 expression in breast cancer. These findings are supported by the recent observation that miR-10b expression decreases as a function of grade in breast cancer (Gee et al., 2008).

Rac1 activity is tightly controlled by several factors in addition to TIAM1. These include GAPs, GDIs, and other GEFs. GAPs, such as RalA binding protein 1 (RalBP1), increase the rate of GTP hydrolysis. GDIs bind to GDP-bound Rac1, thereby inhibiting nucleotide exchange. GEFs – including TIAM1, Trio (Triple functional domain (PTPRF interacting)), Son-of-sevenless-1 (Sos-1) and Pak-interacting exchange factor β (β-Pix) – stimulate the exchange of GDP for GTP, thereby activation Rac1. In addition, Ras family monomeric G-proteins, including K-Ras and H-Ras, act as upstream regulators of Rac1, regulating the activation states of Rac1 in response to growth factor or cytokine stimulation (Bar-Sagi and Hall, 2000; Walsh and Bar-Sagi, 2001). Rac1-dependent migration of carcinoma cells in response to chemoattractant or cell surface receptor signaling, specifically clustering of β1 integrin, is also regulated by cAMP-dependent protein kinase (PKA) (O'Connor and Mercurio, 2001). Such an extensive level of coordinate regulation of Rac1 activation has important implications for the regulation of cancer cell motility. My findings add a new dimension to Rac1 activation that involves miRNA. Given that miRNAs can regulate numerous targets simultaneously, much remains to be learned about Rho GTPases are regulated in cancer.

In addition to Rac1, TIAM1 also acts as a GEF for Rho and Cdc42. RhoA and Cdc42 have been shown to potentiate invasion of fibroblasts (Stam et al., 1997), however, RhoA can also function to impede cell invasion in invasive breast carcinoma due to a reciprocal relationship between RhoA and Rac1

activation (Simpson et al., 2004). Furthermore, both Rac and Cdc42 have been shown to inhibit Rho activity (Sander et al., 1999). Further investigation is necessary to determine whether miR-10b alters the activation status of Cdc42 and RhoA via TIAM1 or Rac1, and whether crosstalk through shared effectors affects cancer cell motility.

Although TIAM1 has been implicated in breast cancer progression, to date no one has examined its expression in breast cancer metastases. It will be important to determine whether expression of TIAM1 is maintained in distant metastases, and if expression is cancer subtype dependent. Based on my findings and others (Adam et al., 2001; Minard et al., 2004), I predict that TIAM1 expression is maintained in metastases and that TIAM1 plays an important role in metastatic spread.

As a key factor in cell motility, TIAM1 may be a promising target for cancer therapeutics. TIAM1 rescues the miR-10b phenotype of decreased cell motility, suggesting that TIAM1 is the primary target of miR-10b. Therefore, targeting knockdown of TIAM1 in cancer may prove to be an efficacious therapeutic strategy. One potential concern is that targeting TIAM1 may disrupt cell-cell adhesion in epithelial cells. Data from TIAM1 knockout mice indicate that loss of TIAM1 impedes Ras-induced oncogenesis, however, these mice develop, grow and reproduce normally (Malliri et al., 2002). This indicates that therapeutics directed against TIAM1 may impede breast cancer cell motility without impairing epithelial cell-cell adhesion. Further investigation is needed to determine whether

knockdown of TIAM1 *in vivo* gives the same migratory phenotype as expression of miR-10b.

Implications for FLT-1

The VEGF/PlGF receptor FLT-1 has also been implicated in both cell motility and breast cancer progression. FLT-1 expression correlates with a high risk of metastasis and relapse in both node negative and invasive breast cancer (Ghosh et al., 2008; Meunier-Carpentier et al., 2005; Mylona et al., 2007; van 't Veer et al., 2002). Furthermore, treatment with anti-FLT-1 antibody or FLT-1-specific inhibitory peptides suppresses breast tumor growth and metastasis (Bae et al., 2005; Luttun et al., 2002; Taylor and Goldenberg, 2007; Wu et al., 2006). Collectively, these data suggest that FLT-1 plays an important role in breast cancer progression. Although FLT-1 functions to promote cell motility in other solid tumors, the function of FLT-1 in breast cancer cell motility has not yet been explored. As shown in Chapter 3, FLT-1 promotes both cell migration and invasion in SUM-159PT breast cancer cells. FLT-1 knockdown using RNAi causes a striking decrease in cell invasion, with a more modest effect on cell migration. This suggests that FLT-1 functions primarily to promote invasion through the basement membrane and into the surrounding tissue, with a lesser role in cell migration through the ECM. To date, one other group has examined FLT-1 function in breast cancer, reporting that FLT-1 promotes cell survival via an internal autocrine signaling pathway (Lee et al., 2007). My own preliminary

observation is that FLT-1 knockdown in SUM-159PT cells has no adverse effects on cell survival, although this will need to be verified experimentally by cell proliferation assays and Annexin-V/PI staining for flow cytometric study of cell death. However, it is possible that the internal autocrine signaling pathway previously identified (Lee et al., 2007) may account for ligand-independent migration that I observed in these cells. Further investigation to examine the localization of FLT-1 in these cells is warranted. If FLT-1 is localized internally in SUM-159 cells, it provides an explanation for the failure of VEGF or PlGF to stimulate SUM-159 cell migration (Chapter 3) and suggests that FLT-1 expression may promote a basal level of signaling that contributes to cell motility.

Expression of miR-10b in SUM-159PT cells causes significant downregulation of FLT-1 protein, while inhibition of endogenous miR-10b in T47D cells increases FLT-1 expression. These data indicate FLT-1 is a target of miR-10b and provide a novel mechanism for regulation of FLT-1 expression in breast cancer. Furthermore, these data provide evidence that miR-10b regulates expression of not one, but two key proteins involved in breast cancer cell motility and breast cancer progression. To determine whether FLT-1 is a direct target of miR-10b, it will be necessary to perform luciferase reporter assays demonstrating the direct interaction of miR-10b with the FLT-1 3'UTR.

FLT-1, generally believed to be a cell surface receptor, is especially attractive as a drug target for blocking breast cancer progression, and is currently under investigation as a target in radioimmunotherapy (Lee et al., 2009). In

addition, anti-FLT-1 monoclonal antibody IMC-18F1 (Wu et al., 2006) is now in Phase I clinical trials in patients with advanced solid tumors who have not responded to standard therapy or for whom no standard therapy is available (National Cancer Institute Clinical Trials database, 2009). I have shown that FLT-1 plays a role in cell migration and invasion in breast cancer, however, further studies are needed to examine downstream signaling pathways as well as other functions of FLT-1 such as cell survival and metastasis.

Other (Novel) miR-10b Targets

Exploration of other miR-10b targets is warranted to further understand the network of targets and signaling pathways regulated by miR-10b. NFAT5 (nuclear factor of activated T-cells 5) is a putative target of miR-10b that has been previously implicated in breast cancer cell motility. NFAT5 is a transcription factor that is expressed in invasive human ductal breast carcinomas and promotes cellular invasion of both breast and colon cancer cell lines (Jauliac et al., 2002). As such, NFAT5 may be another important target regulated by miR-10b in breast cancer. Overexpression of putative miR-10b target and receptor tyrosine kinase EPH receptor A2 (EphA2) is commonly observed in aggressive breast cancer and correlates with a poor prognosis (Brantley-Sieders et al., 2008). Several of these genes have the potential to contribute to the miR-10b phenotype. Thus, miR-10b may regulate a multitude of genes simultaneously in the suppression of cell motility. Other genes of interest, which may have

functions other than cell motility, include putative miR-10b target BACH2 (BTB and CNC homology 1, basic leucine zipper transcription factor 2), which is upregulated in ovarian cancer (Motamed-Khorasani et al., 2007).

Cooperative Regulation with Other miRNAs

Interestingly, both TIAM1 and FLT-1 are predicted to be regulated by other common miRNAs in addition to miR-10b. These miRNAs include miR-302b and miR-17-5p. The function of the miR-302 cluster, which is known to be specifically expressed in embryonic stem cells, has not yet been explored in cancer. However, miR-17-5p has been found to suppress cell proliferation and function as a tumor suppressor in breast cancer (Hossain et al., 2006; Yu et al., 2008). These findings suggest that key target proteins such as TIAM1 and FLT-1 may be regulated by multiple miRNAs simultaneously. An important goal for the future is understanding how miRNAs function as a network, in other words studying the coordinate action of multiple miRNAs on a single miRNA target. Coordinate action of miR-10b, miR-17-5p, and miR-302 on common targets may be necessary for optimal gene regulation during cancer progression. It will be important to determine which miRNAs act on TIAM1 and FLT-1, as well as other common targets.

Given that miR-10b contains the same seed sequence as miR-10 family member miR-10a, and differs from miR-10a by only a single base pair, it will also be important to investigate the role of miR-10a and its targets in breast cancer.

MiR-10a is predicted to have many of the same targets as miR-10b and is therefore likely to play to a similar role in suppression of breast cancer cell motility. Interestingly, previous studies of miR-10a in breast cancer have shown that it regulates HoxD4 in a transcriptional manner (Tan et al., 2009), indicating that miRNA control of transcription may be another potential system for gene regulation in breast cancer. In addition, miR-10a interacts with the 5' untranslated region of mRNAs encoding ribosomal proteins to enhance their translation (Orom et al., 2008), thereby providing a novel mechanism for global protein synthesis. It will be important to determine whether miR-10b is capable of acting in a similar manner to regulate gene expression, as well as whether miR-10a and miR-10b act cooperatively to regulate breast cancer cell motility.

Regulation of miR-10b Expression

MiR-10b is downregulated in breast cancer (Iorio et al., 2005), but the regulation of miR-10b itself has not yet been explored. Important questions that will need to be answered include when, where, and how miR-10b expression is lost during the course of cancer progression. For example, miR-10b expression could be downregulated during the oncogenic transformation of a normal breast epithelial cell to a cancer cell, or it may be lost during the course of cancer progression. If miR-10b is lost during the course of cancer progression, it may be lost only at the invasive front or throughout the primary tumor. Future studies utilizing fluorescence *in situ* hybridization (ISH) with locked nucleic acid probes to

examine expression of miR-10b in archived specimens will help to resolve these questions (Sempere et al., 2007).

It is also important to identify the mechanism for miR-10b downregulation in cancer. Determining the specific subcellular compartmentalization of the precursor and mature forms of miR-10b through ISH may provide insight into the modulation of both miR-10b and its targets. Possible mechanisms of regulation include defects in miRNA biogenesis, promoter methylation, and transcription factor regulation.

MiR-10b is located within the HoxD gene cluster on chromosome 2 at 2q31.1. MiRNAs located in hox clusters have been shown, in general, to inhibit more anterior hox genes ("posterior prevalence phenomenon") (Lempradl and Ringrose, 2008). HoxD10 has been identified as a miR-10b target (Ma et al., 2007). However, I was not able to confirm this result. HoxD10 expression is progressively reduced in epithelial cells as malignancy increases in breast cancer and has been postulated to act as a tumor suppressor (Carrio et al., 2005). Therefore, it is possible that highly aggressive SUM-159PT cells may not express HoxD10. Given that SUM-149 cells were previously shown by our lab and others to express high levels of RhoC (Simpson et al., 2004), it seems unlikely that miR-10b suppression of HoxD10 in these cells leads to induction of RhoC, as reported by Ma et al. (2007). Therefore, the validity of HoxD10 as a target of miR-10b will need to be confirmed. However, as a family of transcription factors, the HoxD genes may play a role in regulating miR-10b at the transcriptional level,

increasing miR-10b transcription in normal tissue and/or repressing transcription in cancer. Interestingly, HoxD10 has recently been shown to regulate miR-7 in various human cancers (Reddy et al., 2008). In breast cancer, the expression of miR-7 is positively regulated by HoxD10, the loss of which increases invasiveness. Furthermore, protein levels of the miR-7 target Pak1 are progressively upregulated whereas those of miR-7 and its upstream activator HoxD10 are progressively downregulated in a cellular model of breast cancer progression from low to highly invasive phenotypes (Reddy et al., 2008). It is possible that HoxD10 regulates miR-10b in the same manner. Future studies are necessary to examine this possibility.

Relevance to Human Breast Cancer

I propose that expression of miR-10b is diminished in breast cancer and that this change in expression is critical to breast cancer progression. In normal breast epithelial cells, miR-10b represses expression of key target proteins, such as TIAM1 and FLT-1. In breast tumor cells, expression of miR-10b is decreased, thereby releasing inhibition of TIAM1 and FLT-1, allowing these proteins to be expressed. Increased expression of TIAM1 activates Rac1, leading to increased cell migration and invasion. Increased expression of FLT-1 also contributes to increased cell invasion, and to a lesser extent, migration. The ability of these cancer cells to migrate through the ECM and invade the basement membrane

encasing the tumor increases the probability of local invasion and metastatic spread, thereby promoting breast cancer progression.

Given the finding that miR-10b suppresses breast cancer cell motility, it is important to consider the significance of this for clinical breast cancer. All of my studies were conducted *in vitro*, therefore, it will be important to extend future studies to *in vivo* systems, such as xenograft models of breast cancer or a transgenic miR-10b knockout, to examine the physiologic relevance of miR-10b to breast cancer progression, and specifically to the processes of local invasion and metastatic spread. The function of miR-10b as a suppressor of cell motility may extend to other solid tumors, such as colon and prostate carcinomas, and therefore the efficacy of miR-10b gene therapy may be universal. The reconstitution of tumor suppressive miRNA, such as miR-10b, has remarkable potential in the treatment of cancer. However, key technical aspects – including optimization of selectivity, stability, *in vivo* delivery, efficacy, and safety – need to be investigated before miRNA can be utilized as a successful therapeutic strategy.

MiR-10b may also prove useful in cancer diagnosis. Expression profiling of miRNAs has been shown to be a more accurate method of classifying cancer subtypes than using the expression profiles of protein-coding mRNAs. Therefore, differential expression of certain miRNAs, such as miR-10b, in various tumors might become a powerful tool to aid in the diagnosis and treatment of cancer. MiR-10b has previously been identified as one of 15 miRNAs in a miRNA

signature able to discriminate between normal breast tissue and breast tumor tissue (Iorio et al., 2005), however, the findings regarding miR-10b as a potential prognostic marker in breast cancer are controversial (Gee et al., 2008; Ma et al., 2007). To resolve this discrepancy, it will be important to examine miR-10b expression in both primary breast tumors and distant metastases according to degree of invasion as well as cancer subtype (i.e. luminal, basal, Her2neu, and normal-like). It is possible that miR-10b plays different roles in these different tumor types, and therefore miR-10b may be an effective therapeutic for some patients but not others.

Discrepancy with Published Data

The data on miR-10b function in breast cancer cell lines that I report in this thesis conflict with the findings published by Ma et al. (2007). To verify my results, I obtained the same miR-10b retroviral plasmid prepared by Ma et al. and used for their studies. In addition, I utilized the same cell line, SUM-149, and conducted migration assays under the same conditions. Briefly, cells were cultured under the same conditions and transduced with a miR-10b retrovirus or empty vector control. Migration and invasion assays were conducted on uncoated or Matrigel coated transwells, respectively, utilizing serum as the chemoattractant. I found that exongenous expression of either a miR-10b precursor or a miR-10b retroviral plasmid suppresses the motility of SUM-159PT and SUM-149PT cells. In contrast, Ma et al. (2007) observed that exogenous

miR-10b enhances the motility of SUM-149PT cells. While I cannot rule out the possibility of acquired cell differences, the accumulated data clearly indicate that miR-10b *suppresses* cell migration and invasion in breast carcinoma cells. Given this result, it is difficult to reconcile the opposing conclusions regarding miR-10b in breast cancer cell motility. However, my data are supported by the recent finding that miR-10b correlates inversely with breast tumor size and grade, but not with cancer metastasis (Gee et al., 2008).

Interestingly, I observed that miR-10b is expressed in an epithelial-like, non-metastatic cell line, but not in an invasive or aggressive metastatic cell line, in direct contrast to previously published results that miR-10b is highly expressed only in metastatic breast cancer cells (Ma et al., 2007). T47D cells, a differentiated epithelial strain derived from a pleural effusion of infiltrating ductal carcinoma (Keydar et al., 1979), express miR-10b. These cells are relatively non-motile *in vitro* and non-metastatic *in vivo*. In contrast, SUM-159PT cells are an aggressive triple negative line (ER-/PR-/Her2-) derived from anaplastic carcinoma. These miR-10b-null cells are metastatic upon orthotopic injection in nude mice (Flanagan et al., 1999). Similarly, miR-10b-null SUM-149PT cells are derived from locally invasive inflammatory breast cancer, a particularly lethal form of breast cancer. These cells are postulated to undergo metastatic spread via a passive mechanism as tumor emboli, but are metastatic nonetheless (Hoffmeyer et al., 2005).

I also question the purported role of miR-10b in inducing RhoC expression because our laboratory had reported previously that SUM-149PT cells, which were shown to lack miR-10b expression by both us (Fig. 1) and Ma et al. (2007), express relatively high levels of RhoC (Simpson et al., 2004). RhoC is overexpressed in breast cancer and has been postulated to be a novel marker for aggressive breast carcinomas with metastatic ability (Kleer et al., 2005; Kleer et al., 2002), such as the SUM-149PT and SUM-159PT breast cancer cell lines. Furthermore, HoxD10 expression is progressively reduced in epithelial cells as malignancy increases in breast cancer and has been postulated to act as a tumor suppressor (Carrio et al., 2005). I believe it is possible that aggressive, invasive breast cancer cell lines such as SUM-149PT and SUM-159PT do not express HoxD10 protein. Western blot analysis will be necessary to confirm this hypothesis.

In conclusion, the data presented in the preceding chapters contributes to our understanding of the regulation of cell migration and invasion in breast cancer. Future research in this area will undoubtedly provide insight into the role of miRNAs in breast cancer and perhaps lead to valuable diagnostic tools and clinical therapies for breast cancer treatment.

APPENDIX

This appendix contains publications to which I have contributed, but that are not included in the main body of my thesis.

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Apigenin Suppresses Cancer Cell Growth through ERβ[1]

Paul Mak, Yuet-Kin Leung†, Wan-Yee Tang†, Charlotte Harwood* and Shuk-Mei Ho†*

*Department of Surgery, University of Massachusetts Medical School, Worcester, MA 01605, USA; †Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, OH 45267, USA

Abstract

Two flavonoids, genistein and apigenin, have been implicated as chemopreventive agents against prostate and breast cancers. However, the mechanisms behind their respective cancer-protective effects may vary significantly. The goal of this study was to determine whether the antiproliferative action of these flavonoids on prostate (DU-145) and breast (MDA-MB-231) cancer cells expressing only estrogen receptor (ER) β is mediated by this ER subtype. It was found that both genistein and apigenin, although not 17β-estradiol, exhibited antiproliferative effects and proapoptotic activities through caspase-3 activation in these two cell lines. In yeast transcription assays, both flavonoids displayed high specificity toward ERβ transactivation, particularly at lower concentrations. However, in mammalian assay, apigenin was found to be more ERβ-selective than genistein, which has equal potency in inducing transactivation through ERα and ERβ. Small interfering RNA-mediated downregulation of ERβ abrogated the antiproliferative effect of apigenin in both cancer cells but did not reverse that of genistein. Our data unveil, for the first time, that the anticancer action of apigenin is mediated, in part, by ERβ. The differential use of ERα and ERβ signaling for transaction between genistein and apigenin demonstrates the complexity of phytoestrogen action in the context of their anticancer properties.

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Keywords: Phytoestrogens, genistein, ERα, apoptosis, cancer chemoprevention.

Introduction

Flavonoids present in soy, fruits, and vegetables have been implicated as chemopreventive agents for a variety of cancers [1–3]. The best-studied flavonoid is genistein, an isoflavone abundant in soy. The beneficial effects of dietary soy are supported by epidemiological observations that countries with high soy consumption, such as China and Japan, have lower incidences of prostate and breast cancers than countries with little or no soy consumption [4]. In experimental models, dietary genistein reduces the incidence of prostate [5] and breast cancers [6,7]. Cellular and molecular mechanisms underpinning the anticancer effects of genistein cover a broad array of cellular processes, including suppression of cell growth, angiogenesis, oxidative stress, and tissue responses to estrogens [8]. Genistein is also recognized as a phytoestrogen because it binds to estrogen receptors (ERs) and exhibits both weak estrogenic and antiestrogenic activities.

Recently, the antitumor action of another dietary flavonoid, apigenin (4′,5,7-trihydroxyflavone), has received growing attention. It is abundantly present in leafy plants and vegetables (e.g., parsley, artichoke, basil, and celery) [9], but its production from manufacturers comes from extracts of dried flower heads of *Matricaria chamomilla* L. (http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/Apigenin.pdf; accessed July 8, 2006). As a nutriceutic, apigenin is most widely used in treating anxiety and sleep disorders because it has been shown to possess sedative, antispasmodic, and spasmolytic actions [10]. The flavonoid also holds great promise as a chemopreventive agent for a variety of cancers. It exhibits significant activity against UV-induced DNA damage and thus may protect against skin cancer [11,12]. It inhibits the growth of a variety of human cancer cells, including leukemia and breast, colon, skin, thyroid, and prostate cancers [13,14]. Reported mechanisms associated with its antitumor action include induction of cell cycle arrest and apoptosis through a tumor necrosis factor–induced NFκB-mediated apoptosis pathway [14,15], attenuation of the phosphorylation of epidermal growth factor receptor and MAP kinase [16], promotion of HER-2/neu degradation [17], and activation of the intrinsic apoptosis pathway [18,19]. However, the likelihood that apigenin acts as an estrogen or antiestrogen has not been considered as a mechanism mediating its antitumor action.

It is now known that the actions of estrogens, antiestrogens, and phytoestrogens are mediated by two ER subtypes (ERα and ERβ) whose expression levels vary dramatically among different organs or cell types [20]. The two receptors regulate different sets of biologic functions and incite dissimilar

Address all correspondence to: Shuk-Mei Ho, PhD, Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, OH 45267.
E-mail: shuk-mei.ho@uc.edu

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responses within the same cell type or tissue. Furthermore, it has become apparent that the actions of these receptors vary dramatically, depending on whether they exist alone or together in a cell [21,22]. Because genistein and other phytoestrogens have been shown to preferentially use ERβs over ERαs as signaling mediators [23–26], it is reasonable to anticipate that apigenin exhibits a similar preference for estrogenicity.

The present study seeks to test the hypothesis that apigenin-induced cancer cell death is mediated by ERβ and neither by ERα nor androgen receptor. The prostate cancer cell line DU-145 [27,28] and the breast cancer cell line MDA-MB-231 [29,30] were chosen as study models because they both express only ERβ. The growth-inhibitory action of apigenin on these cancer cell lines was examined in the presence or in the absence of small interfering RNA (siRNA)–mediated downregulation of the receptor. The transactivation activities of apigenin at the estrogen-responsive element (ERE), through ERβ, were compared to those mediated by ERα. Comparisons were also made between apigenin, 17β-estradiol (E_2), ICI-182,780 (ICI), and genistein to elucidate the estrogenic properties of apigenin.

Materials and Methods

Reagents and Chemicals

Yeast synthetic dropout media were obtained from Clontech (Takara Bio, Palo Alto, CA). All steroids, phytoestrogens, and etoposide used in this study were purchased from Sigma (St. Louis, MO). The antiestrogen ICI was kindly supplied by Zeneca Pharmaceuticals (Cheshire, UK). The Beta-Glo Assay System was purchased from Promega (Madison, WI). DNA restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). Antibodies against human ERα (sc-8005) or ERβ (sc-8974) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines and Culture Conditions

Two human prostate cancer cell lines (DU-145 and PC-3), a breast cell line (MDA-MB-231), and an embryonic kidney cell line [human embryonic kidney (HEK) 293] were obtained from American Type Culture Collection (Manassas, VA). DU-145 and HEK293 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, nonessential amino acids, and penicillin/streptomycin (Invitrogen, Carlsbad, CA). PC-3 and MDA-MB-231 cells were maintained in DMEM/F12 or MEM-α medium, respectively, supplemented in the same fashion. Cells were maintained at 37°C and 5% CO_2.

Cell Viability Assay

Cell viability assays were conducted in phenol red–free medium supplemented with 5% charcoal-stripped serum, nonessential amino acids, and penicillin/streptomycin. Cells were plated at 4×10^3 cells/well in 200 μl of phenol red–free medium in 96-well plates. Stock solutions of compounds in dimethyl sulfoxide (DMSO) were stored at 10 mM and mixed with fresh medium to achieve a final concentration of 10 nM E_2, 1 μM ICI, or 20 μM genistein or apigenin. Cells were allowed to adhere for 24 hours, at which time the medium was removed and replaced with media containing one of the above agents. Control cultures received a medium containing the vehicle DMSO. Treatment was performed in triplicate and repeated at 48-hour intervals. On the sixth day, cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; inner salt] assay, as described previously [31]. The medium was aspirated, and the cells were washed once with 200 μl of Hanks balanced salt solution. Ten microliters of MTS reagent (CellTiter 96 Aqueous One Solution Reagent; Promega) and 50 μl of medium were added to each well. Following 1 to 4 hours of incubation at 37°C and 5% CO_2, absorbance was recorded by μQuant microplate reader (Biotek, Winooski, VT) at a wavelength of 490 nm.

Caspase-3 Assay

DU-145 or MDA-MB-231 cells were plated into six-well plates at 3×10^5 cells/well in 3 ml of phenol red–free medium and allowed to attach for 24 hours. Cells were treated with 10 nM E_2, 1 μM ICI, and 20 μM etoposide, genistein, or apigenin. Control cultures were treated with vehicle alone (DMSO). Treatment time was 48 hours. The presence of apoptotic cells was determined by measuring caspase-3 using the BD ApoAlert Colorimetric Caspase-3 assay (Clontech), according to the manufacturer's instructions.

Knockdown of ERβ by Specific siRNA

Culture conditions for DU-145 ($ER\alpha^-$ and $ER\beta^+$) and MDA-MB-231 ($ER\alpha^-$ and $ER\beta^+$) have been described previously [27,29]. Cells were plated in 96-well or 6-well plates for MTS assay or RNA extraction, respectively, 1 day before transfection with siRNA oligonucleotides. Cells were transfected with 50 nM siRNA oligonucleotide using Lipofectamine2000 (Invitrogen), according to the manufacturer's protocol. siRNA against ERβ was purchased from Dharmacon's siGENOME SMART pool selection (Lafayette, CO) and was proven to knock down ERβ expression at the mRNA level by at least 75%. Negative control siRNA, an siCONTROL pool, and transfection siRNA control (siTOX) were included to ensure the specificity and transfection efficiency of siRNA. Twenty-four hours after transfection, cells were incubated with 10 or 20 μM apigenin or genistein for another 72 hours and subsequently analyzed for cytotoxicity with MTS assay. To correct for nonspecific toxic effects of siRNA, cell viability after treatments with a phytoestrogen (siESR2 + phytoestrogen or siCTL + phytoestrogen) was normalized against its respective no-phytoestrogen-treatment control (siESR2 alone or siCTL alone).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis of ERβ Transcript Levels

Total RNA was isolated from transfected cells with TRI reagent (Sigma), according to the manufacturer's protocol. The integrity of RNA was confirmed by denaturing gel, as described previously [32]. Total RNA (4 μg) from each sample

was reverse-transcribed to cDNA by Superscript II reverse transcriptase (Invitrogen). Semiquantitative RT-PCR was performed using Platinum *Taq* polymerase (Invitrogen) with ERβ-specific primers [33]. The forward and reverse primers for β-actin have been described previously [27].

Construction of Yeast Strains

The yeast expression (YEpE10) and reporter (YEp-vERE) plasmids for ERα were kindly supplied by Dr. Tauseef Butt (LifeSensor, Inc., Malvern, PA). These plasmids were used to transform the protease-deficient yeast strain BJ2168 according to standard protocol [34]. This double-transformant yeast strain was grown in synthetic dropout medium (−TRP −URA). The yeast strain expressing ERβ in the presence of an estrogen-responsive reporter plasmid (YEp-vERE) has recently been described [33]. This double-transformant yeast strain was grown in synthetic dropout medium (−LEU −URA).

Yeast-Based Transcription Assays

To study the hormone responsiveness of ERα or ERβ, double yeast transformants carrying an expression plasmid for ERα or ERβ and a reporter plasmid (YEp-vERE) were selected for ligand-dependent transcriptional activity. Expression of ERβ was analyzed by Western blot analysis [35] using an N-terminal–specific H-150 polyclonal antibody (Santa Cruz Biotechnology). Because CUP1 promoter was moderately leaky in this experiment (data not shown), addition of copper was not necessary to induce ERβ expression. All selected transformants were grown in a synthetic dropout medium in a 96-well plate overnight at 30°C, either in the absence (control) or in the presence of steroids and phytoestrogens (0.01 nM–10 μM). A ligand-dependent transactivation assay was performed using the Beta-Glo Assay System (Promega), according to the manufacturer's protocol. Induction of the reporter gene (luciferase activity in relative light units) was measured by the Victor 2 system (Perkin Elmer, Wellesley, MA).

Transactivation Activity of ERα and ERβ in a Mammalian System

Mammalian expression vectors ERα and ERβ were gifts from Dr. Leigh C. Murphy (University of Manitoba, Winnipeg, Canada) [36]. The luciferase reporter plasmid carrying 3X vitellogenin ERE was kindly provided by Dr. Craig Jordan (Fox Chase Cancer Center, Philadelphia, PA) [37].

HEK293 cells at a density of 1.5×10^4 ml^{-1} were seeded onto a 24-well plate. Regular culture medium was replaced by phenol red–free DMEM with 5% charcoal-stripped serum. The cells were allowed to adopt an estrogen-free environment for 48 hours before transfection. Vectors expressing ERα or ERβ + ERE luciferase and β-galactosidase were transfected into the cells using Lipofectamine Plus (Invitrogen). After 24 hours of transfection, E_2 (100 pM and 1 nM), ICI (10 nM and 1 μM), apigenin (100 nM and 1 μM), and genistein (100 nM and 1 μM) were applied to the culture. In separate experiments, cells were treated with E_2, apigenin, or genistein in the absence or in the presence of the antiestrogen ICI. Luciferase reporter assay was performed as suggested in the Bright Glo Luciferase assay kit (Promega) to determine transactivation activity after 24 hours of treatment with hormones/compounds. Activities of β-galactosidase were measured by a β-gal assay kit (Promega) to normalize the transfection efficiency of each well.

Statistical Analysis

Data were expressed as mean ± SD. The statistical significance of the difference in means among treatment groups was determined with Systat software (Student version 6.0.1; SPSS, Chicago, IL) for one-way ANOVA followed by Tukey post hoc analyses. $P < .05$ was considered as a statistically significant difference between the two groups.

Results

Apigenin and Genistein Suppressed Cell Growth through Induction of Apoptosis in DU-145 and MDA-MB-231 Cells

The effects of E_2 (10 nM), ICI (1 μM), genistein (20 μM), or apigenin (20 μM) on the growth of DU-145 and MDA-MB-231 in charcoal-stripped medium were examined. At the doses tested, E_2 exerted no impact on the growth (Figure 1*A* and *B*) or the apoptosis (Figure 1*C* and *D*) of either cell lines, whereas ICI induced a small reduction in cell number and an increase in caspase-3 activation in DU-145 cells when compared with control vehicle (DMSO), but not in MDA-MB-231 cells. On the contrary, apigenin and genistein effectively suppressed the growth of both cell lines. Apigenin was more effective than genistein in suppressing DU-145 cell growth (Figure 1*A*) but exhibited potency equal to that of genistein in inhibiting MDA-MB-231 proliferation (Figure 1*B*). The growth inhibition induced by the two phytoestrogens paralleled their abilities to induce caspase-3 activation. Apigenin and genistein were equally effective in causing caspase-3 activation in both cell lines (Figure 1*C* and *D*).

Differential Transcriptional Activation of ERα and ERβ in Response to Estrogens and Phytoestrogens in Yeast Cells

The transcriptional activities of E_2, ICI, genistein, and apigenin through ERα and ERβ were evaluated in a yeast system. As expected, E_2 was found to transactivate better through ERα than through ERβ (Figure 2*A*). Interestingly, although ICI is a well-recognized antagonist in mammalian cell studies, it behaved as an agonist in yeast cells, transactivating more effectively with ERα than with ERβ (Figure 2*B*). This finding is in agreement with findings in a previous report using yeast reporter assays [38]. In contrast to E_2 or ICI, apigenin and genistein behaved as ERβ-selective ligands at low phytoestrogen concentrations. At higher ligand concentrations, they still elicited much higher transactivation activities through ERβ than through ERα (Figure 2*C* and *D*).

Differential Transactivation of ERα and ERβ in Response to Estrogens and Phytoestrogens in Mammalian Cells

The HEK293 cell line was chosen as a mammalian reporter assay system due to its low background as a transcription assay and the absence of endogenous ERα and

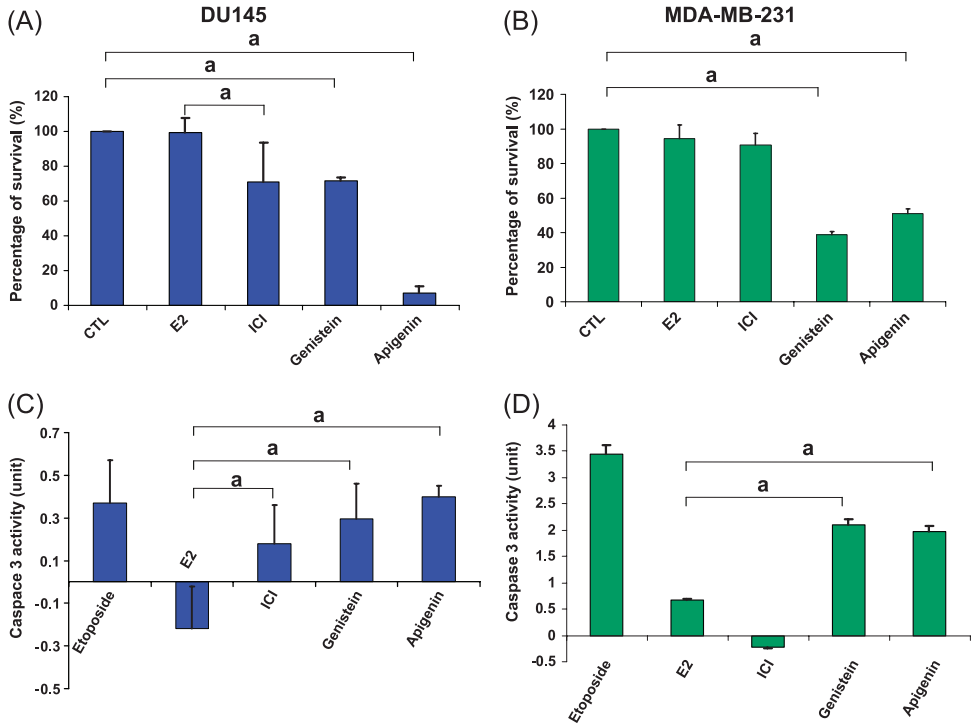

Figure 1. *Effects of genistein or apigenin on the proliferation of (A) DU-145 and (B) MDA-MB-231 cells. Cells were treated with 10 nM E_2, 1 μM ICI, 20 μM genistein, or 20 μM apigenin for 72 hours. Control (CTL) cultures were treated with solvent vehicle in a charcoal-stripped serum-supplemented medium. Cell viability was determined by MTS assay, as described in the Materials and Methods section. (C and D). Induction of relative caspase-3 activity by genistein or apigenin in (C) DU-145 and (D) MDA-MB-231 cells. Cells were treated with E_2, ICI, genistein, and apigenin at the concentrations described above. E_2 and etoposide serve as negative and positive control, respectively, for this experiment. Caspase-3 activities are normalized with respect to the control vehicle (DMSO). Twenty micromolars of etoposide was used as positive control. Data represent the averages (histograms) of three separate experiments, with the standard deviation indicated. [a]Statistically significant difference between the treatment group and the control group (control vehicle for A and B; E_2 for C and D) at $P < .05$.*

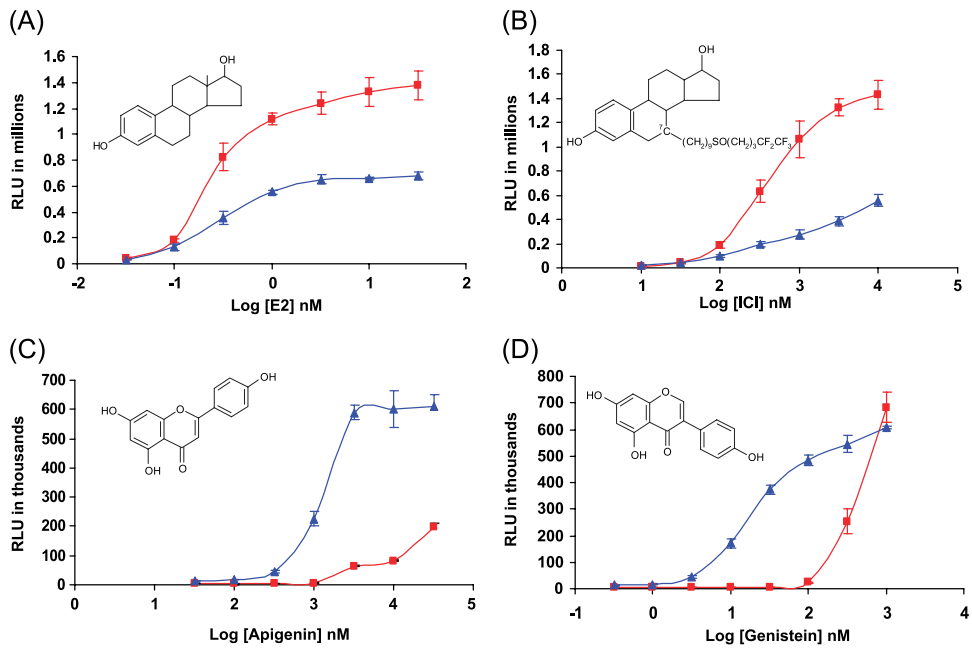

Figure 2. *Differential transcriptional activation of ERα and ERβ1 in yeast. Yeast strains harboring the expression vectors for ERα (red square) or ERβ1 (blue triangle) and the vitellogenin ERE reporter plasmid were incubated with an increasing concentration of E_2 (A), ICI (B), apigenin (C), or genistein (D). After 24 hours of incubation at 30°C, Beta-Glo assays were performed. Luciferase activity (relative light units) was recorded by a luminometer (Victor 2 system). Each point represents an average of triplicates with standard deviations.*

ERβ expression [33]. When physiological concentrations of E_2 (100 pM and 1 nM) were used, this ligand elicited potent dose-dependent transactivation responses through ERα (Figure 3*A*). In contrast, E_2 elicited only low levels of transactivation through the ERβ. Contrary to results obtained in yeast systems, the antiestrogen ICI was inactive in these mammalian transcription assays regardless of its action being mediated by ERα or ERβ. In this regard, the results are consistent with the widely accepted notion that ICI is an estrogen antagonist for mammalian cells. The two phytoestrogens clearly exhibited agonistic actions in mammalian reporter assays. Apigenin was a weak agonist at a lower concentration (100 nM) compared to genistein, which achieved maximal transactivation through ERα and ERβ at the same concentration. At a higher concentration (1 μM), apigenin exhibited clear ERβ selectivity; it induced significantly higher transactivation through ERβ than through ERα. Thus, these data appear to corroborate those observed in yeast-based transcription assays, except for those related to ICI, which acts as an agonist in yeast assays. Importantly, all transcriptional activation activities induced by E_2, apigenin, and genistein through ERβ could be attenuated efficiently by the coincubation of cell cultures with 1 μM ICI, indicating that they were mediated by ERβ (Figure 3*B*). Similar results were obtained when this experiment was conducted with ERα expressing HEK293 (data not shown).

ERβ Plays a Role in Apigenin-Induced Cancer Cell Growth Inhibition

The relative efficacy of siRNA against ERβ (ERβ siRNA), negative control siRNA (siCONTROL pool), and transfection siRNA control (siTOX) in knocking down ERβ transcripts in transfected cells was determined by RT-PCR. As shown in Figure 4*A*, ERβ transcripts were reduced by > 85% in DU-145 and MDA-MB-231 cells, whereas the other siRNA (siCONTROL pool and siTOX) did not affect the expression of ERβ transcripts when compared to cultures without siRNA transfection (control cultures were treated with Lipofectamine alone). Transfection efficiency in these experiments was estimated as 70%.

Subsequently, DU-145 and MDA-MB-231 cells were examined for cell viability after treatment with phytoestrogens. For both cell lines, apigenin (10 and 20 μM) induced a dose-dependent reduction in cell growth (Figure 4*B* and *C*), whereas genistein caused only cell growth inhibition at the high dose (20 μM) (Figure 4*D* and *E*). Importantly, the antiproliferation effects of apigenin on DU-145 and MDA-MB-231 were effectively abrogated by transfecting the cells

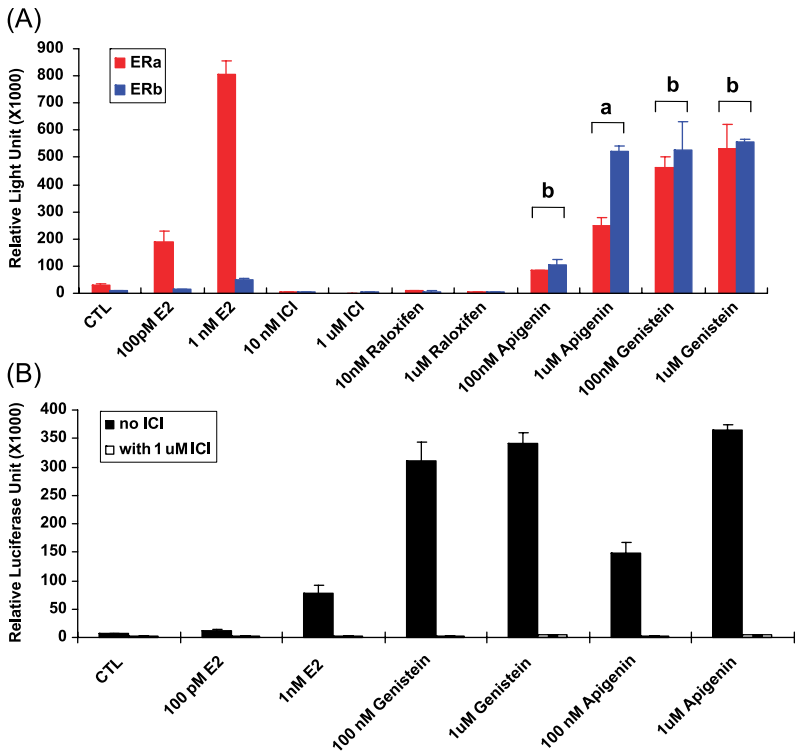

Figure 3. *(A) Transcriptional activation of ERα and ERβ1 in mammalian cells. HEK293 cells were transfected with vectors expressing ERα (red bar) or ERβ1 (blue bar), β-galactosidase, and a vector carrying 3X ERE luciferase reporter using Lipofectamine Plus, as described in the Materials and Methods section. After 24 hours, cells were treated with E_2, ICI, apigenin, genistein, or solvent vehicle (CTL) and subsequently analyzed for luciferase activity. Data represent three separate experiments with standard deviations.* [a]*Statistical significance between the treated group and untreated controls at P < .01.* [b]*No significant difference between the two groups. (B) Effects of ICI on the ligand responsiveness of ERβ1 in HEK293 cells were seeded onto 24-well plates and transfected with ERβ1 expression vector, 3X ERE luciferase reporter, and β-galactosidase expression plasmids, as described above. Cells were treated with solvent vehicle (CTL), E_2, genistein, or apigenin in the absence (black bar) or in the presence (white bar) of 1 μM ICI and subsequently analyzed for luciferase activity. Data represent three separate experiments with standard deviation.*

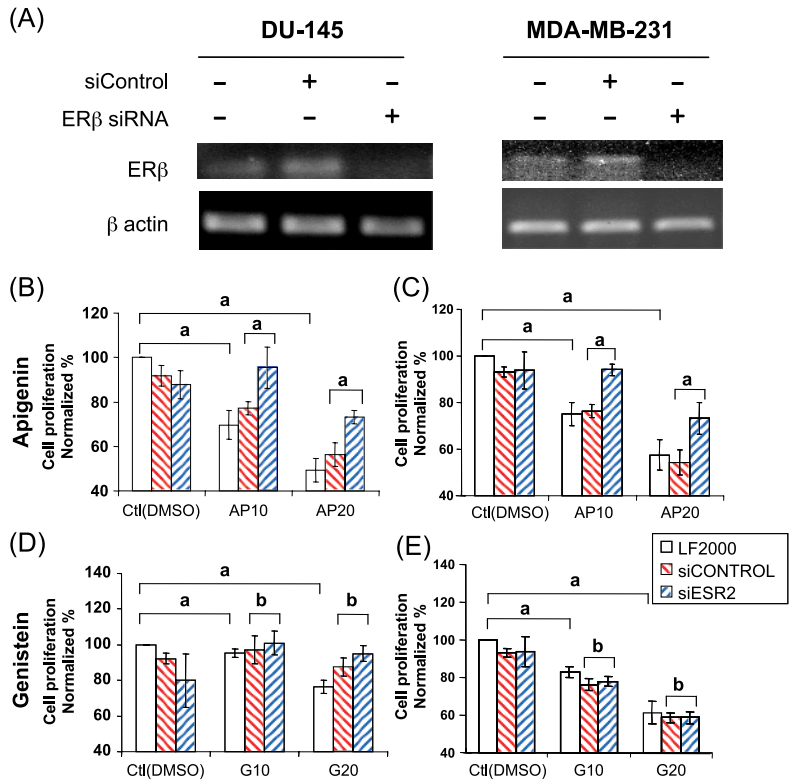

Figure 4. *(A) ERβ1 knockdown experiments in DU-145 and MDA-MB-231 cells. Cells were transfected with 50 nM siRNA against ERβ1 or scrambled siRNA (siCONTROL), as described in the Materials and Methods section. After 72 hours of incubation, total RNA was extracted and semiquantitative RT-PCR was performed using specific primers against ERβ1 and β-actin. Similar results were obtained from two separate experiments. (B–E) Antiproliferation effects of apigenin or genistein on ERβ1 knockdown DU-145 and MDA-MB-231 cells. siRNA against ERβ (siESR2)– and siRNA scrambled control (siCONTROL)–transfected cells were incubated with 10 or 20 μM apigenin (B and C) or genistein (D and E) for 72 hours and subsequently analyzed for cytotoxicity by MTS assay, as described in the Materials and Methods section. Control groups (Lipofectamine2000) without siRNA transfection were treated with vehicle (CTL). The viability of the cells with apigenin or genistein treatment (siESR2 + phytoestrogen or siCTL + phytoestrogen) was normalized to values obtained in cultures treated similarly but without phytoestrogens (siESR2 alone and siCTL alone). The cell viability in each treatment group was calculated as a percentage of the value found in untreated controls without siRNA transfection. Data represent the average of three separate experiments, with standard deviation indicated. [a]The mean of the treated group was statistically different from that of untreated controls at P < .05. [b]No significant difference between the treated group and the untreated control.*

with ERβ siRNA (Figure 4*B* and *C*), but not by transfection of siCONTROL (Figure 4*B* and *C*), siTOX (data not shown), or ERα siRNA (data not shown). In contrast, genistein-induced cancer cell growth suppression was not reversed by ERβ siRNA transfection (Figure 4*D* and *E*).

Discussion

The two flavonoids genistein and apigenin have been studied extensively for their antitumorigenic activities in various cancers, including prostate and breast cancers [8,14,15, 39,40]. Although many different mechanisms of action have been proposed [8,13,16–18,39–42], a connection between the anticancer effects of these flavonoids and ERβ has not been described. Several reports have demonstrated that interactions between ERα and ERβ could significantly alter the transactivation activities of each receptor and biologic outcome [21,22]. Therefore, we have chosen the two cancer cell lines DU-145 and MDA-MB-231, which express only ERβ [27,29], to compare the antiproliferative effects of two flavonoids. Furthermore, because transcriptional activities of a nuclear receptor could be markedly altered by post-translational protein modification and coregulator interaction in mammalian cells [43], we have evaluated the transcriptional potentials of these two flavonoids in yeast and mammalian reporter assays. Because yeast cells are devoid of transcription coregulators and most posttranslational protein modification pathways, basal transcriptional activities could be obtained for comparison with activities in a mammalian reporter system (HEK293). In this study, we found that the exposure of DU-145 or MDA-MB-231 cells to either flavonoid elicited suppression of cell growth and activation of caspase-3—a hallmark of apoptosis. In regard to the concentrations of genistein or apigenin used and the extent of growth inhibition/apoptosis induction in cancer cells, our findings were consistent with those reported by others [8,19,39,44–46]. For example, genistein has been reported to trigger apoptosis in breast cancer through calcium-dependent and calpain/caspase-12–dependent pathways [47]. In both yeast and mammalian reporter assays, the two flavonoids were found to act as agonists at the ERE and to exhibit ERβ selectivity for transactivation when compared to E_2, which transactivates most effectively through the ERα. Between the two flavonoids, apigenin was found to be more selective with transactivations by ERβ than was genistein, which was equally potent in eliciting transactivation through

the two ER subtypes. The major contribution of our study, however, resides in the finding that the antiproliferative/proapoptotic effect of apigenin is apparently mediated by ERβ, whereas that of genistein, at least in these two cell models, does not involve the receptor. To the best of our knowledge, this study, which used siRNA knockdown of ERβ, is the first to demonstrate the involvement of this ER subtype in the antiproliferative/proapoptotic effect of apigenin.

In DU-145 cell cultures, apigenin-induced growth inhibition was noticeably greater than that induced by genistein, whereas in MDA-MB-231 cell cultures, both flavonoids exhibited comparable potency. Consistent with our previous observations, E_2 was found to exert little effect on the growth of DU-145 cells, whereas ICI induced a modest inhibitory action [27]. The action of the antiestrogen on the prostate cancer cell line was shown to be dependent on ERβ [27] and may involve a crosstalk with the NFκB signaling pathway [48]. Morrissey et al. [18] suggested that apigenin-mediated apoptosis in DU-145 may not involve ERs. Their conclusion was reached based on the cotreatment of apigenin-exposed DU-145 cells with ICI and their observation of a lack of attenuation of the apigenin effect. Our findings offer a possible explanation for their observation. Because ICI could exhibit its antiproliferative/proapoptotic on DU-145 cells per se [27], the addition of ICI to apigenin-treated cells would unlikely block the proapoptotic effects of apigenin. In this study, our use of siRNA to specifically knock down ERβ proves to be a better approach to demonstrating the involvement of the receptor in apigenin action.

In MDA-MB-231 breast cancer cells, as expected [49], E_2 did not stimulate cell growth and ICI had no action on proliferation/apoptosis. These findings are consistent with previously reported findings that the lack of ERα in this cell line makes it insensitive to estrogen stimulation in terms of cell proliferation. In contrast, both genistein and apigenin are effective antiproliferative/proapoptotic agents for this ERα$^-$ cell line. In the case of apigenin, its action was found to be mediated by ERβ in this study. If our observation in MDA-MB-231 could be extended to ERα$^-$ breast cancers, apigenin might have clinical utility in the chemoprevention of the recurrence of these cancers [50].

In the present study, we demonstrated that apigenin and genistein, when compared to E_2, exhibit markedly different transactivation potencies through the two ER subtypes. E_2 effectively induces ERα-mediated transcription but only triggers minimal transactivation through ERβ. In contrast, apigenin and genistein are excellent ERβ-mediated transactivators. This property of the two flavonoids is most noticeable in yeast reporter assays, which lack modulations from endogenous transcriptional coregulators or cofactors. Even in mammalian cell assays (HEK293 cells), both flavonoids are highly effective in eliciting ERβ-mediated transactivation. A key difference between the two resides in the strong selectivity of apigenin for ERβ-mediated transactivation, whereas genistein is equally effective in activating ERα-mediated and ERβ-mediated transcription. Collectively, findings from yeast and mammalian reporter assays suggest that apigenin is an ERβ-selective ligand, whereas genistein can activate both receptor subtypes. This conclusion is corroborated by a recent report that found genistein to have a higher binding affinity toward ERα than does apigenin [51].

The differences between these two flavonoids as phytoestrogens could be related to several key attributes that define the mode of estrogen action. Generally speaking, phytoestrogens have weak binding affinities for both ER subtypes, but they bind ERβ better than ERα [52]. However, a greater binding affinity of a phytoestrogen for a specific ER subtype does not always correlate with its ability to better transactivate gene expression through that receptor [53,54]. Other important factors that determine selectivity for an ER subtype include the ability of the phytoestrogen to create a high-affinity coregulator-binding pocket by the correct positioning of helix 12 within the ligand-binding domain of the ER–ligand complex [55]. In this regard, phytoestrogens have been shown to confer ERβ with coregulator-recruiting affinity higher than that of ERα [23,56]. Although most soy isoflavones, including genistein, are believed to exert their actions primarily through ERβ signaling [57,58], recent studies [59,60] have raised doubts about this assumption. Our data from HEK293 reporter assays support these doubts as genistein was found to be equally effective in eliciting transactivation through either ER subtype. This lack of selectivity of genistein for ERβ signaling may pose a limit to its use as a chemopreventive agent for breast cancer because its ERα activity may post concern for increasing the risk of recurrence of ERα breast cancers, undesirable uterotrophic activities, and thromboembolic disorders. It is well established that estrogen action on the uterus and liver is exclusively mediated by ERα signaling [61].

The most intriguing finding of the current study is that the siRNA-mediated knockdown of ERβ blocked only the growth-inhibitory effect of apigenin—not that of genistein—on DU-145 and MDA-MB-231 cells. This finding suggests that the anticancer growth effect of apigenin—but not of genistein—involves ERβ. Indeed, pharmacological dosages of genistein have been shown to trigger cytotoxic activities through ER-independent pathways, such as inhibition of tyrosine kinase and topoisomerase [62]. Although apigenin has been shown to elicit pleiotropic effects on a variety of pathways that mediate antitumor actions [14–17,19], our report is the first one to associate it with ERβ signaling. More recently, genistein and apigenin have been demonstrated to act as estrogen agonists in ERα/β$^-$ MCF-7 and T47-D cells by acting through ERα [29]. Whether apigenin could suppress cell growth in other ER$^+$ cancer cell lines remains to be determined as both DU-145 and MDA-MB-231 cell lines express only ERβ but not ERα [27–29]. It has been reported that ERα can heterodimerize with ERβ and alter its transactivation activity [21,22]. It is therefore logical to expect cancer cells that express both ER subtypes to respond to apigenin in a manner different from what has been demonstrated in DU-145 and MDA-MB-231 cells. It is surprising to find that the genistein-induced anticancer cell growth action in DU-145 and MDA-MB-231 cells was not affected by siRNA-induced downregulation of ERβ. Although the anticancer action of genistein on prostate and breast cancers

has been widely reported, it remains uncertain whether it is mediated through ERβ. In this study, we showed that, at least in two cancer cell lines that express only ERβ, the anticancer effects of genistein are mediated through mechanisms not involving this receptor.

In summary, we have demonstrated the preferred usage of ERβ by apigenin as a mediator in suppressing the growth of DU-145 and MDA-MB-231 cells. Overall, apigenin, when compared to genistein, has a much stronger selectivity for ERβ than for ERα. Continued efforts placed on this area of research might provide important insights on the synthesis of highly selective ERβ agonists for anticancer intervention.

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